--chk conjugat ~ folder

31 prec. limitation on prod. clm.

10/076,031

09/25/03

(21) charge modified prot

prot. = targeting moiety, conjug. ~ targeting moiety or diagnostic/therapeutic agent

36 charge from: phosphate, phosphonate, sulfate, nitrate, borate, silicate, $CO_3^{=}$, COOH

EP 329,184

p13 anion-forming agents react ~ 1° NH_2 on lysine

(a) → p14 succinic anhydr. (clm 36)

↳ NHS, thiphenyl ester, tetraf'l esters + thioesters

(b) react ~ Arg: glyoxal, phglyoxal, cyclohexanedione

→ acidic shift caused by

con 6,358,490 last

5,283,342 1st no chg. mod. here f. 4/09/92

chg. modif. here → 5,578,287 + maybe before (G 11/23/93 in ABN applics.)

N.M. no description of i.w.? (p.13-14)

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(71) Applicant: NEORX CORPORATION
410 West Harrison Street
Seattle Washington 98119(US)

(72) Inventor: Morgan, Alton C., Jr.
803 Driftwood Place
Edmonds Washington 98020(US)
Inventor: Sivam, Gowsala P.
23504 - 97th Place West
Edmonds Washington 98020(US)
Inventor: Abrams, Paul G.
2125 First Avenue No. 1602
Seattle Washington 98121(US)
Inventor: Srinivasan, Ananthachari
11420 - 109th Avenue N.E.
Kirkland Washington 98033(US)
Inventor: Reno, John M.
2452 Elm Drive
Brier Washington 98036(US)
Inventor: Fritzberg, Alan R.
16703 - 74th Place West
Edmonds Washington 98020(US)
Inventor: Priest, John H.
7307 Heather Way
Everett Washington 98203(US)
Inventor: Anderson, David C.
2415 Thorndyke, No. 301
Seattle Washington 98199(US)

(74) Representative: Brown, John David et al
FORRESTER & BOEHMERT
Widenmayerstrasse 4/I
D-8000 München 22(DE)

(54) Antimers and antimeric conjugation.

(57) There is disclosed an antibody and antibody-drug conjugate for targeting drug delivery as well as a class of chemicals, termed an "antimer." The antimer is designed to "fit" the drug by combining multiple non-covalent interactions between functional groups on the drug and opposing functional groups on the antimer. The net result on the antibody-antimer-drug conjugate is a drug stably bound to the antimer so as not to dissociate during in vivo administration, but not so tightly bound to allow drug dissociation from the conjugate without significant loss of activity and retaining the drug's ability to bind to a higher affinity site on the or within the target cell.

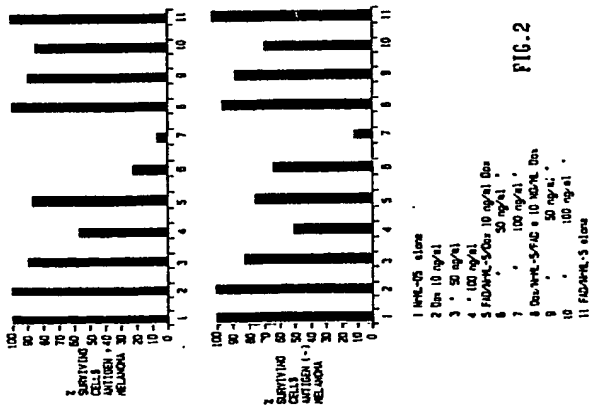


FIG.2

EP 0 329 184 A2

ANTIMERS AND ANTIMERIC CONJUGATION

Technical Field

The present invention relates to compounds, termed "antimers," which function to bind drug molecules non-covalently to a carrier or antibody with such affinity that there is little dissociation of the drug from a carrier or antibody and with no loss of drug potency, but with a reduction in toxicity. The compounds can be used to conjugate a drug to a delivery system, such as an antibody or other targeting protein. Antimers can occur in nature or can be designed ("designer antimer") to bind specifically to a drug by using compounds with similar structures but with one or more "opposing" functional groups, capable of forming non-covalent bonds, opposite each drug functional group and thereby complexing with the drug via multiple non-covalent interactions.

Background of the Invention

There has been considerable interest in the "magic bullet" approach to cancer therapeutics. Recent efforts have been devoted to the conjugation of chemotherapeutic neoplastic drugs to specific antibodies, such as monoclonal antibodies, to produce conjugates which can selectively target tumor cells while sparing normal tissues. Different classes of agents have been considered for this application. These include beta- and alpha-emitting isotopes, plant and bacterial toxins, and a variety of antineoplastic drugs, including intercalating agents, antimetabolites, alkylating agents, and antibiotics. It is desirable to conjugate chemotherapeutic drugs to antibodies for the following reasons:

1. It has recently been shown that up to 1,000-fold more drug can be delivered to tumor cells when conjugated to an antigen-specific monoclonal antibody than is possible by the addition of free drug.

2. Pleiotropic drug resistance may arise following treatment with one of a number of chemotherapeutic drugs, resulting in inducing resistance to drugs of several classes. The mechanism(s) of this resistance are not entirely known, but it is known that this resistance can be partially overcome by antibody targeting of drugs.

3. Even though current chemotherapeutic drugs are active against only some of the major tumor types, the response rate in drug-insensitive tumor types may be increased by antibody-mediated delivery.

4. Many dose-limiting toxicities, which are now seen with chemotherapeutic drugs, may be reduced by conjugation to an antibody. A decrease in toxicity with at least equal efficacy would give a superior product, and the product would have a higher therapeutic index.

To create conjugate with a drug and an antibody, the drug may be directly linked to the antibody through nucleophilic substitution of certain groups on the antibody (e.g., lysines, carboxyl, or sulfydryl), or the drug may be conjugated to the antibody via hetero- or homo-bifunctional cross-linkers. Linker groups may be small organic compounds or peptides substituted with chemical linkers for conjugation. Large carriers have also been used containing linker groups and offer the advantage of being able to bind many drug molecules to a single antibody. Examples of carriers are the polymers of lysine and glutamic acid, dextran, and the polypeptide albumin.

Drugs have been thus far conjugated to antibodies or carriers only be using covalent bonds. See Blair and Ghose, J. Immunol. Meth. 59: 129-144, 1983. Covalent bonds can be further subclassified into non-metabolizable and metabolizable bonds. Metabolizable bonds are those that undergo hydrolysis, releasing the drug under conditions present within or around cells, such as low pH, a reducing environment, or through proteolysis. An example of metabolizable covalent bonds which are useful are those that are sensitive to the low pH environment of endosomes within a cell. See Shen and Ryser, Biochem. + Biophys. Res. Commun. 102: 1048-1054, 1981. After the drug-antibody conjugate binds to the cell, it may be internalized by a pathway that places it in an endosome where the conjugate is subjected to a low pH environment. The hydrolysis of the conjugate's covalent bond releases free drug, where it may then exert its cytotoxic activity.

A non-metabolizable bond can also result in active drug conjugates. However, the r sulting conjugates with non-metabolizable bonds generally have reduced drug potency as compared with those conjugates formed with metabolizable bonds. This is because intracellular processing via proteolysis does not rel ase the drug as efficiently as metabolizable bonds. In addition, the drug, if released, is usually in a form different from the native drug and has reduced cytotoxic potency.

Covalent drug-antibody conjugates have been made where th drug is conjugated directly to th antibody and also wher the drug is covalently bound to a carri r before conjugation to the antibody. See U.S. Patent No. 4,507,234, Garnett and Baldwin, Cancer Res. 46: 2407-2412, 1986. Direct conjugation consists of a drug's being conjugated to residues within th antibody molecule, including, for xampl , lysine and glutamic acid amino groups, sulfhydryl groups and sugar residues within oligosaccharid chains. An important limitation of this direct conjugation approach is that the antibody may be exposed to harsh conjugation conditions, that may denature the antibody causing more rapid clearance from the serum after injection. Unless the direct conjugation is site directed (e.g., at the carbohydrate or sulfhydryl groups), the immunoreactivity of the conjugate may be compromised. Even when combined with site-direction, direct conjugation can still result in nonselective (i.e., kill antigen-positive and antigen-negative cells with approximately equal potency) and poor target localization, due to the nature of the agent conjugated to the antibody. As an example, ricin A chain conjugated via site-directed sulfhydryl groups to antibodies is rapidly taken up by liver phagocytes due to mannose receptors for carbohydrate on the ricin A chain. This can also occur with highly lipophilic drugs because lipophilic drugs in free drug form must have some means for interacting with cells to be effective. One such mechanism for lipophilic drugs is insertion into the cell membrane lipid bilayer. If the direct drug-antibody conjugate is formed with a metabolizable covalent bond, this bond can often be metabolized at other sites within the body, such as within the blood, liver, spleen, and other organs.

The indirect method of conjugation first requires the coupling of a drug to a carrier, generally via a linker group. The carrier is then conjugated to the antibody, via a heterobifunctional linker, that can be first conjugated to the carrier and then activated following drug conjugation. One advantage of this indirect conjugation route is that large numbers of drug molecules may be linked to an antibody for delivery to the target site. However, large numbers of drug molecules linked to an antibody may also lead to enhanced nonspecific uptake due, for example, to the lipophillicity of the drug. The indirect conjugation approach does not expose the antibody to the harsh conditions of conjugation, as the chemical manipulations are usually performed on the carrier and not the antibody. Furthermore, the carrier can enhance the solubility of the drug conjugate.

Direct or indirect conjugation of a drug to an antibody creates a stable conjugate that can arrive at the target site with a minimum of dissociation of the drug. One needs, however, to couple this property with a mechanism of selective release of drug for maximal potency.

Selective release may be exploited at three levels. The first is intracellular release within the tumor cell. The best examples of this form of release are pH-sensitive and reducible bonds which, upon intracellular processing of the conjugate, break down to release free drug. This requires binding and internalization of the conjugate prior to drug dissociation. Intracellular internalization of the conjugate requires that the conjugate either enter the cytoplasm or be taken up into an endosome, or lysosome. Internalization rates with monoclonal antibodies to antigens of solid tumors is slow. Thus, a drug conjugate requiring such a process for release of active drug will not be highly potent. In addition, not all internalized conjugate undergoes appropriate intracellular processing for release of active drug. Conjugates that are processed into lysosomes are probably degraded and some drugs will be inactivated. Conjugates processed in the endosomes or into the cytoplasm have the opportunity to release their drugs and allow drugs access to the intracellular target.

A second site for the selective release of drug from conjugate is the plasma membrane. One example of the plasma membrane release mechanism is referred to in United States Patent No. 4,671,958. In this case, conjugate that is once bound to tumor cells activates complement, which causes the proteolytic degradation of sensitive peptide linkages, to which the drug is bound and releases it in free form.

A third level for drug release would be at the tumor site, but before the conjugate is bound to the tumor cell. This third form of release requires a drug-antibody linkage that would take advantage of certain differences between tumor and normal tissue extracellular milieu. None have been developed to date.

Covalent drug conjugates discussed above comprising cytotoxic or antineoplastic drugs covalently conjugated to an antibody with or without the use of a carrier through linker groups, in a site- or non-site-directed manner, suffer from a number of problems. First, covalent conjugation of drug to antibody requires derivatization of the drug to produce a form of the drug capable of being conjugated to groups in the antibody or carrier. This typically results in a reduction of the drug's cytotoxic activity or potency, due to chemical modification of its functional groups. For some drugs, exposure to the conditions for derivatization may be sufficient to inactivate the drug. For others, the derivatization is not well enough controlled so that groups important for th drug's cytotoxic activity are chemically modified, although these groups are not the primary targets of the procedure. The use of labile bonds, such as pH- sensitive bonds, may overcom part of this problem, but may still result in relatively slow release of the drug at the targeted site or release of

the drug in a modified, less active form.

Extracellular release of the drug from the conjugate, as described in United States Patent No. 4,671,958, overcomes the internalization and intracellular processing problems associated with conjugates. The drug, however, still must be derivatized appropriately in order for it to be covalently bound to carbohydrate residues within the antibody molecule, either directly or through a carrier-mediated system. In addition, the rates of release of the drug will be governed by the half-life of the antibody on the plasma membrane of the tumor cells and by the rate of complement fixation of the antibody. This process is a handicap with most murine monoclonal antibodies (the type most often used), that have little or no ability to fix human complement.

The current generation of immunoconjugates of drugs and antibodies suffer from the additional problem of poor selectivity. This problem of decreases selectivity can be assessed by testing drug conjugates in vitro against antigen-positive and antigen-negative cells. Antigen-positive cells are usually killed at drug-conjugate concentrations tenfold or less lower than antigen-negative cells. This is true, for example, for anthracycline conjugates. Conjugates of the same antibody and a plant or bacterial toxin will, by contrast, typically show 3 to 4 logs of selectively. It thus seems apparent that the cytotoxic drug itself has additional mechanisms for interacting with cell membranes, and that this leads to nonselective cytotoxicity. Moreover, there is often more than one drug molecule conjugated to an antibody molecule with each drug molecule being capable of nonselective cellular interactions. Thus, there is a considerable need in the art to improve the selectivity of drug immunoconjugates. This can provide improved delivery in vivo to tumor sites as well as decreased normal tissue uptake.

Summary of the Invention

The above-identified problems can be addressed and the current generation of immunoconjugates improved by the use of non-covalent binding methodologies for conjugating drugs to antibodies or to other carrier molecules. The use of non-covalent binding does not expose the drugs to harsh derivatization conditions and thereby does not compromise the potency of the drug. The use of non-covalent binding methodologies produce sufficiently high affinity of binding of the drug for targeting, but also sufficiently labile that the cytotoxic drug may transfer at the target site to drug acceptors on the cell surface or within the tumor cell. Indeed, one of the advantages of the non-covalent approach is that it allows discrete titration of the affinity of interaction to produce the desired balance of drug binding and release. The conjugate envisioned by the present invention comprises a carrier-drug or targeting protein conjugate for slow drug release or targeted drug delivery, respectively: comprising a targeting protein such as an antibody or antibody fragment, or carrier molecule; a moiety termed an "antimer" wherein the antimer is covalently bound to the antibody or carrier; and a drug non-covalently complexed to the antimer. In a separate configuration, drug can be first bound through covalent bonds to antibody or carrier and then complexed with antimer to improve the cytotoxic selectivity of the killing. The antimer can be found in nature or specifically designed. (Such specifically designed antimers are hereinafter referred to as "designer antimers").

Non-covalent association of a drug with a carrier protein or antibody is random and heterogenous in binding affinities, and generally results in only low levels of bound drug. The less stably bound drug is considered undesirable due to the increased potential for premature release and increased risk of host toxicity and a reduced ability to localize to tumor sites. The present invention provides for an antimer that is specifically designed to fit the drug molecule and undergo multiple non-covalent interactions with a drug to enhance its binding affinity to antibody or a carrier and to provide a conjugate stable enough to arrive at target sites with most of the drug still bound.

This invention also comprises a soluble complex of drug and antimer. This preformed complex is administered as a pharmaceutical material to decrease the toxicity of a cytotoxic regimen or to provide a mechanism for the slow release of free drug. The antimer alone may also be injected at the local site of drug injection to prevent toxicity, such as, the extravasation of doxorubicin around an intravenous site of injection.

Description of the Drawing

Figure 1 depicts a doxorubicin molecule aligned with a naturally occurring antimer, flavin adenin dinucleotide. The alignment of the molecules represents the pi-pi bonding or stacking which the two molecules can undergo. Potential hydrogen bonds of this drug/antimer complex are also shown. In addition, ionic binding can occur between the amino group of the drug and th phosphate groups of the antimer. Th multipl non-covalent interaction results in a high affinity complex. Th antimer can be modified at the R group with typical nucleophilic groups like active esters or mallmides, for conjugation to antibody or carri r.

Figure 2 compares the potency and selectivity of covalent and non-covalent antibody (NrML-5)-/antimer (FAD)/drug (Dox for Doxorubicin or adriamycin). The assay reported in Figure 2 is an in vitro cytotoxicity assay for both antigen positive melanoma cells (top) and antigen negative melanoma cells (bottom). The monoclonal antibody NrML-5 is specific for the melanoma antigen. The bar graphs show percentage survival as determined by MTT dye uptake and metabolism (Example 5).

Figure 3a depicts a best-fit titration curve for 50 μM doxorubicin and varying concentrations of the peptide EC(MIANS)GC(MIANS)EGGC(Acm) in μM.

Figure 3b shows the binding at absorbance wavelengths of 565 nm and 580 nm, for a best-fit dissociation constant at 48 μM peptide concentration.

Detailed Description of the Invention

Prior to setting forth the invention, it is helpful to set forth certain definitions.

Antimer:

The term "antimer" is not presently used in the art but is introduced here to describe the present invention. The term "mer" is used in the art to describe a chemical species which is related to another chemical species; for example, the term "isomer" refers to a chemical compound that has the same number and kind of atoms as another molecule, but with different spatial configurations. The term "antimer" refers to a molecule that has a form opposite and complementary to that of another molecule. In this patent application, the term "antimer" is used to mean a molecule having functionalities that are opposite and complementary in structure to a drug molecule. Preferably, the antimer and drug will have similar planar ring structures, but with opposing functionalities. Opposing functionalities on the antimer will include groups for hydrogen and ionic binding and other non-covalent interactions, with or without electron poor (e.g., groups giving rise to acidic protons) or electron rich (e.g., unshared electron pairs on heteroatoms and anionic groups, such as carboxylates) groups on the antimer to increase the pi binding. Furthermore, the opposing functional groups on the antimer will be sterically oriented in proper three-dimensional alignment such that the functional group on the drug can interact with the opposing functional group on the antimer in proper steric orientation.

Functional Group:

Functional group is that part of a molecule that can interact and form an association or bond with another group. For example, electron-rich groups, electron-poor groups, dipoles with hydrogen bonding potential, as well as ionic moieties, are examples of functional groups. Referring to the structure of doxorubicin in Figure 1, the keto group, the hydroxy group, the methoxy group, and the amino group are examples of functional groups on the doxorubicin molecule.

Antineoplastic Agent:

An antineoplastic agent is a drug molecule that has cytotoxic activity by virtue of its ability to inhibit DNA, RNA, protein synthesis, or some other essential cellular function, ultimately leading to cell death.

Non-Covalent Bond:

A non-covalent bond is d fined as an ionic bond, a hydrogen bond, a pi-pi bond, hydrophobic

interactions and van der Waals interactions.

Covalent Bond:

A covalent bond is defined as the formation of a sigma bond between two organic molecules.

Electron-Rich Groups:

An electron-rich group contains excess electrons or available basic non-bonding pairs of electrons. Examples of electron-rich groups include but are not limited to carboxylates, sulfinates, phenoxides, and groups containing heteroatoms with unshared pairs of electrons, such as oxygen, sulfur, and nitrogen.

Electron-Poor Groups:

An electron-poor group is electron deficient as in catons or containing atoms of high electronegativity. Electron-poor groups include but are not limited to protons bound to heteroatoms thereby being acidic, ammonium nitrogen atoms, and nitrogroups.

Steric Location:

The steric location of a functional group on an antimer is that position where the antimeric opposing functional group should be located to line up opposite the drug's functional group and allow for a non-covalent bond to form between the drug's functional group and the antimer's opposing functional group.

Carrier:

A carrier is a polypeptide, polymer, or protein such as poly-1-lysine, poly-1-glutamate, polymeric dextran, or albumin. A carrier is used to bind drug to antibody or targeting protein to increase the loading without reducing immunoreactivity of the antibody.

Targeting Protein:

A targeting protein comprises any protein moiety that can specifically bind to a target cell or target site. Examples of targeting proteins, include antibodies, antibody fragments (Fab, $F(ab')_2$, and Fab'), monoclonal antibodies, monoclonal antibody fragments and peptide hormones that can bind to specific cellular receptors. The targeting protein serves to direct the complex or conjugate to a specific target site or a specific group of target cells.

Linker Group:

A small peptide or organic molecule with hetero or homo bifunctional linkages for conjugation of drug to a carrier or antibody.

Complementary Groups:

Functional groups capable of interacting with each other to produce attraction froces between two molecules. An electron-poor and an electron-rich group is an example of complementary groups.

Briefly stated, the present invention is an antimer, an antimer/drug complex, a carrier/antimer/drug conjugate, a targeting protein/antimer/drug conjugate, a targeting protein/carrier/antimer/drug conjugate, a targeting protein/drug/antimer complex, a targeting protein/carrier/drug/antimer complex and a method of

designing or producing an antimer wherein an antimer can be identified in nature or synthesized that will undergo multiple, non-covalent interactions with a drug. Though each interaction is relatively weak on its own, when combined, they produce a strong bond to th drug. The non-covalent antimer binding to drugs is, in many ways, analogous to drug-receptor site interactions, that combines hydrophobic, ionic and hydrogen binding to produce stable and selective binding of a drug to its receptor. The antimer molecule uses concerted, multiple, non-covalent interactions to produce stable complexes or conjugates of drugs. The stable complex of drug non-covalently bound to an antimer which is, in turn, covalently bound to a targeting protein. This is accomplished by first producing a molecule that is "antimeric," or similar in structure but having opposing and complementary functionalities, to the drug that is to be conjugated. The opposing and complementary functionalities of the antimer are sterically oriented on the antimer molecule to orient to the functionalities of the durg. This usually results in a similar spatial configuration of the structures of the drug and the antimer.

Preferably, the antimer and the drug will have a planar ring structure as nearly identical to each other as possible. This will allow for "stacking" and pi-pi or charge-transfer interactions. These interactions alone are insufficient to produce high-affinity binding, and thus opposing complementary functional groups are situated around the planar ring to interact via hydrogen or ionic bonding with functional groups of the drug. Similarly, in addition, if electron-rich groups are present on the drug, electron-poor groups can be situated on the planar ring of the antimer so as to enhance the pi-pi or stacking between drug and antimer.

One form of a non-covalent bond is a hydrophobic (pi-pi) bond formed between ring structures. Preferably, the ring structures have a similar configuration. Planar rign structures are examples of similar configurations. Hydrogen bonding can occur between a negative dipole, such as -C=0, and a positive dipole, such as OH. Hydrogen bonding is formed between groups with heteroatoms, containing basic unshared pairs of electrons, such as oxygen as in C=0 carbonyl, and groups with cations, such as substituted amines and acidic protons on heteroatoms. Ionic, non-covalent bonds are formed between anionic groups, such as phosphates, phosphonates, sulfonates and other groups with strongly acidic hydrogen atoms and cationic groups, such as NH^{+}_{3}. This list is not meant to be limiting as functionalities may vary in their ability to undergo non-covalent interactions with a drug because of the context of ring structures on which functionality is found.

Often, drugs such as doxorubicin cause extravasation or local necrosis as a form of toxicity at the site of injection. The simultaneous or following administration of an antimer to doxorubicin will bind to the free drug at the local injection site and function to reduce local extravasation of free drug. Similarly, other locally necrotic drugs can have local toxicity reduced by administration of the antimer.

In designing an antimer to bind non-covalently to a specific drug, the overall affinity of the drug for the antimeric structure can be adjusted by increasing or decreasing the number of possible non-covalent interactions between drug and antimer. Generally, the binding has to be approximately $\geq 10^6$ moles/liter in order for the drug/antimer to be sufficiently stable for in vivo delivery, yet the affinity must be low enough for ultimate transfer of the drug to target acceptor sites. The number of groups on the antimer that can undergo non-covalent interactions can be modified by organic synthesis of rings with different numbers of groups or by simple oxidation or reduction of existing antimers. An example is that of flavin adenine dinucleotide, a natural antimer to doxorubicin, which has the potential for three hydrogen bonds per flavin ring in its reduced state but only one hydrogen bond in its oxidized state.

For conjugation, FAD is modified at the indicated R group (See examples). Designer antimers for other drugs are shown in Examples 9-14. In addition, the FAD antimer can be used to bind other known chemotherapeutic drugs with similar ring structures to doxorubicin. Examples include the anthracycline derivatives morpholino and cyano morpholino doxorubicin, epirubicin, actinomycin D, ellipticine and mitomycin C.

The initial non-covalent interaction of the drug and antimer can, if necessary, be enhanced by preforming the drug/antimer complexation in the presence of a dehydrating agent. Water molecules will interfere with pi-pi and hydrophobic interacting. Thus, removal of water initially enhances these binding interactions. Once formed, the stability of the complexes become more resistant to rehydration. Table 1 below lists useful dehydrating agents for promoting drug-antimer interactions and a corresponding concentration range in percentage v/v for optimal interaction of the drug and antimer.

resulting complex generally exhibits more stabl drug binding than th above-described antimer compl xes in which a drug is bound through interactions with only one planar ring structure. This drug carrier system should be useful for aromatic drugs used in treating a variety of diseases, including cancer.

One example of this mbodiment of the inventi n involves oligopeptide sequences comprising two or more amino acids with aromatic side chains. By "aromatic side chains" is meant a side chain comprising at least one aromatic ring. The aromatic side chains are spaced to permit binding of an aromatic drug through interactions (including intercalation) with two of the side chains. A targeting protein may be engineered to contain the desired oligopeptide, using known protein engineering or recombinant DNA techniques. Alternatively, an oligopeptide may be attached to a targeting protein (e.g., using a cross-linking reagent), preferably after drug binding. The resulting conjugates are useful for delivering the drug to a desired target site in vivo.

The oligopeptide may by synthesized, using known peptide synthesis techniques, and may include both natural and the so-called "unnatural" amino acids. The oligopeptides are designed to non-covalently bind the drugs by intercalation of the drug between aromatic side chains of the synthetic peptide or by other interactions of the drug with the peptide side chains. Charged side chains may be included elsewhere in the oligopeptide to make the entire complex more water soluble, and perhaps assist in drug binding. The amino acids in the peptide sequence may be chosen to manipulate the charge density and secondary structure to overcome severe solubility problems plaguing earlier efforts to attach aromatic drugs to antibodies. Including repeating units of a drug intercalating or binding sequence allows the oligopeptide to bind multiple drug molecules, thereby loading the targeting protein with multiple drug molecules for enhanced therapeutic efficacy.

The type and position of side chains in the oligopeptide are chosen according to the structure of the drug to be bound. The drug may be any drug that contains at least one aromatic ring, such as one of the above-described aromatic drugs, including those presented in the examples below. The side chains on the peptide backbone should have at least one planar ring structure that is similar to a ring in the drug to be bound. If the drug contains multiple rings, the side chains preferably also comprise similar multiple rings for tighter drug binding. When bound through interactions with the oligopeptide's side chains, the drug may be in a position in which the rings of the drug are either at an angle to or parallel to similar rings in the side chains, so that the aromatic rings on the peptide side chain bind face to face or edge to face or face to edge with the aromatic rings of the drug molecule. Other peptide side chains may be positioned nearby to assist in drug binding through opposite charge interactions. The negatively charged amino acids, glutamic acid or aspartic acid, may be employed to assist in binding the drug doxorubicin (which contains an NH_3 + group), for example.

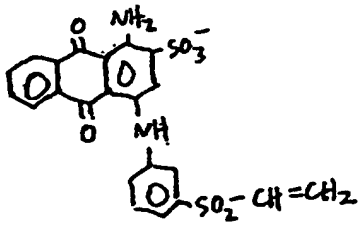
The oligopeptide may include naturally occurring amino acids that have side chains comprising at least one aromatic ring, e.g., tryptophan, phenylalanine, and tyrosine. Alternatively, an oligopeptide may be synthesized to comprise any other appropriate aromatic ring-containing side chains on the peptide backbone, such as 9-fluorenylmethoxycarbonyl (FMOC), pyrene, rhein, uniblue A, acridine ICR191, histidine-N-dinitrophenyl, or benzyl groups, or even the aromatic portion of the drug of interest. The side chains are chosen for similarity to the structure of the drug to be bound. Aromatic compounds known to precipitate the drug, indicating complex formation, also may be used as side chains (e.g., propranolol for the drug doxorubicin).

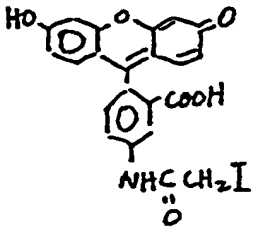
As one alternative, the side chains may be attached after synthesis of an oligopeptide sequence. The use of orthogonal protecting groups in synthesis of oligopeptides comprising two or more of the same amino acids will allow synthesis of binding units with different side chains on each of the same amino acid residues, by protecting them with groups which are deblocked under different conditions. Examples of commercially available orthogonal groups include p-methylbenzyl and acetamidomethyl for cysteine, and 2,6-dichlorobenzoxycarbonyl, FMOC, or trifluoroacetyl for lysine.

Conjugatable aromatic groups which can be easily attached to cysteine, lysine, aspartic acid, glutamic acid, serine, threonine, or arginine residues in a peptide chain have been described by Richard Hargland in Handbook of Fluorescent Probes and Research Chemicals (1985), available from Molecular Probes, Eugene, Oregon. For example, an aromatic compound comprising an alkyl iodide or maleimide group may be reacted with a cysteine residue in an oligopeptide, thereby attaching the aromatic compound to the peptide backbone as a side chain. Aromatic compounds including but not limited to those comprising aromatic isothiocyanates, sulfonyl chlorides, succinimidyl esters, dichlorotriazinyl groups, or alkyl halide nitrobenzoxadiazole derivatives may be reacted with lysine residues. Aromatic compounds containing amine groups may be attached to aspartic acid or glutamic acid residues using carbodiimide coupling reagents. Aromatic compounds containing gly xal, phenyl glyoxal, or dicarboxaldehyd groups are reactive with arginine residues.

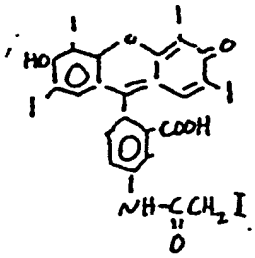
Examples of som of the many such aromatic compounds which may be attached as side chains to certain amino acid residues are the following:

Thiol Reactive (for Attachment to Cysteine Residues)

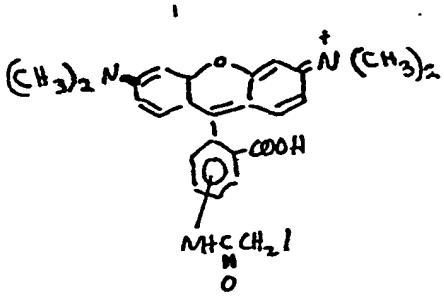




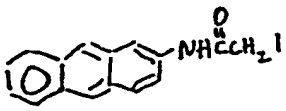
5-iodocetamido-fluorescein



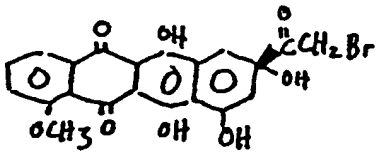
erythrosin-5-iodoacetamide



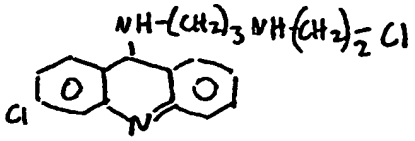
tetramethylrhodamine 5 (or 6)-iodoacetamide



2-anthracene iodacetamide

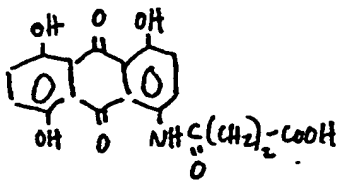


brominated daunomycin aglycone

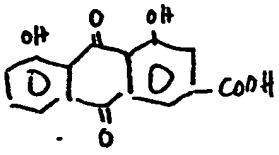


acridine ICR 191

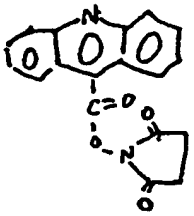
Amine Reactive (Lysine Residues)

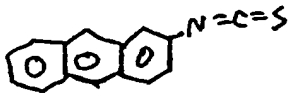


leucoquinizarin derivative

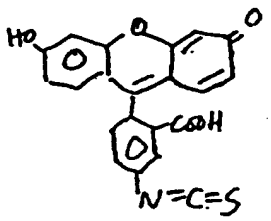


rhein



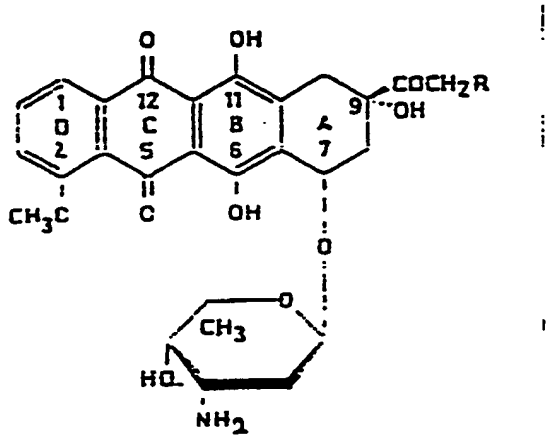


2-anthracene isothiocyanate



fluorescein-5-isothiocyanate

These aromatic compounds may be suitable for non-covalent binding of the anthracycline antibiotics doxorubicin and daunorubicin, which are widely used in cancer therapy and have the following structure:



doxorubicin: R = OH

daunorubicin: R = H

Other aromatic compounds which may be attached to a peptide as a side chain are purines, pyrimidines, or analogs thereof. Appropriate longer oligopeptides may incorporate the cognate bases (i.e., guanine, cytosine, thymine, and adenine), which, after refolding and hydrogen bonding, might mimic double-stranded extended DNA for effective intercalation. Doxorubicin is among the drugs known to intercalate within DNA; thus the synthetic peptide would be acting as a DNA analog for purposes of intercalation.

Individual oligopeptide binding units may be combined into longer peptide carriers to increase the stoichiometry of non-covalently bound drug per antibody. If peptide carriers are insoluble when drugs such as doxorubicin are bound, solubility may be increased both by using more soluble aromatic side chains on the peptide or by including more charged amino acid residues per binding unit, such as glutamic acid or aspartic acid. Peptides are more likely to form secondary structures as their length increases, and a regular structure such as an alpha helix or beta sheet may interfere with drug binding by forcing the binding unit side chains apart. Two or more consecutive glycines may be inserted between binding units to conformationally accommodate individual units, due to the conformational flexibility of the glycines, which tend to break up regular secondary structure. Alternatively, prolines may be inserted between binding units to break up regular secondary structure. A more soluble extended peptide conformation may also be achieved by insertion of two or more glutamic acid residues between drug-binding units to allow chain extension by charge repulsion.

Peptides with consecutive glutamates may be hydrolyzed in lysosomes by glutamate hydrolase after entry of the antibody conjugate into cells, resulting in release of the drug if these residues are important for ion pair formation. Likewise, exposure to lysosomal carboxypeptidases may also enhance drug release after internalization. Another possibility involves insertion of consecutive alanines which may be susceptible to lysosomal elastase hydrolysis.

The oligopeptides may be synthesized using any of a number of known procedures. When desired, additional side chains may be attached to certain amino acid residues after synthesis of the oligopeptide, as described above. Peptide amides can be made using 4-methylbenzhydrylamine-derivatized, cross-linked polystyrene-1% divinylbenzene resin and peptide acids made using PAM (phenylacetamidomethyl) resin (Stewart et al., "Solid Phase Peptide Synthesis," Pierce Chemical Company, Rockford, Ill., 1984). The synthesis can be accomplished either using a commercially available synthesizer, such as the Applied Biosystems 430A, or manually using the procedure of Merrifield et al. (Biochemistry 21:5020-31) 1982, or Houghten (PNAS 82:5131-35, 1985). The side chain protecting groups are removed using the Tam-Merrifield low-high HF procedure (Tam et al., J. Am. Chem. Soc. 105:6442-55, 1983).

The peptid can be extracted with 20% acetic acid, lyophilized, and purified by reversed-phase HPLC on a Vydac C-4 Analytical Column using a linear gradient of 100% water to 100% acetonitrile-0.1% trifluoroacetic acid in 50 minutes. The peptide is analyzed using PTC-amino acid analysis (Heinrikson et al., Anal. Biochem. 136: 65-74, 1984). After gas-phase hydrolysis (Meltzer et al., Anal. Biochem. 160:356-61, 1987), sequences are confirmed using the Edman degradation or fast atom bombardment mass spec-

troscopy.

The oligopeptide may be designed to comprise an amino acid residue (g nerally at the N- or C-terminus of the peptide) through which th oligopeptide can be joined to a targeting protein, such as cysteine or lysine. When necessary, this residue may be orthogonally protected, and deprotected just before or during reaction with the protein. An example would be (nitropyridenesulfenyl) cystein . The oligopeptide may be attached to a targeting protein (preferably after drug binding) using any of a number of known bifunctional cross-linking reagents. The choice of cross-linking reagent depends on the amino acid sequence of the oligopeptide. If the oligopeptide contains a lysine residue, amine-reactive, bifunctional cross-linking reagents such as bis(sulfosuccimidyl) suberate may be used. Alternatively, a water-soluble carbodiimide coupling reagent may be used for form bonds between a free amino group on one reactant (i.e., the oligopeptide or the targeting protein) and a COOH group on the other reactant.

The following examples are designed to illustrate the concept of "antimerism." The examples use the drug doxorubicin, whose structure is illustrated in Figure 1 as the model neoplastic drug. Doxorubicin is chosen for these illustrative purposes because it is an approved drug that is effective against a number of tumors; it is widely available; and its chemotherapeutic mode of action, its pharmacology and its pharmacokinetics have been studied. Additionally, doxorubicin is also often used as a model chemotherapeutic drug in studies of drug interactions with biological compounds.

The following examples are offered by way of illustration and not limitation.

Example 1

An Antimer for Doxorubicin

In many of the examples, a naturally occurring antimeric structure is used. This structure binds to the drug doxorubicin. Flavin adenine dinucleotide (FAD) (see Figure 1) in its reduced state has antimeric functionalities which hydrogen bond to the carbonyl and hydroxyl groups of doxorubicin and undergo ionic interactions between the negative phosphate groups on the FAD and the amino group on the daunosamine sugar, and pi bonding between the planar rings. Accordingly, FAD is an antimer to doxorubicin.

Example 2

Preformed Method of Antimeric Drug Conjugation

In this approach, drug is first complexed with an "activated" antimer and then conjugated to antibody. The antimer is first derivatized with a nucleophilic group such as a maleimide, which can bind to reduced sulfhydryl groups on the antibody molecule. This conjugation is initiated by the step of mixing doxorubicin with FAD in an aqueous, neutral pH buffer (which is slightly hypotonic). The antimer is used at a concentration to initially promote 1:1 complexes between drug and antimer (equimolar offering). Initial binding of the drug and antimer may be promoted by the addition of a dehydrating agent, such as ten percent ammonium sulfate. The drug is added at relatively high concentrations, such as 2 to 4 mg/ml. This results in a larger proportion of the antimer being complexed by multiple non-covalent interactions with the drug, with a small proportion of precipitated drug.

Upon complexation, the hydrogen bonding, ionic bonding, and pi-pi interactions stabilize the complex. Following complexation, the complex is offered to antibody at 10- to 100-fold molar excess of drug/antimer to antibody at room temperature for 1 hour. Antibody, such as a monoclonal antibody, is prepared for conjugation by reduction with dithiothrietal (DTT) See Example 4).

Unbound drug/antimer is then removed by dialysis or g l filtration. Six to ten drug/antim r com plexes per antibody will be bound with direct conjugation of 15-40 complexes via a carri r such as albumin.

Example 3

Post-Formed Method of Antimer/Drug Conjugation

In one example, FAD is coupled directly to antibody. Thus 1 mg of antibody is mixed with 2 mg FAD (5 mg/ml). The solution is warmed to 37°C in a water bath, and 2 mg of EDCI (1-ethyl-3-(3-dimethyaminopropyl)carbodimide) at 10 mg/ml is added. The pH is adjusted to approximately 6.0 with 1N HCl and the reaction stirred for approximately 20 minutes at 37°C. 0.7 ml of 0.05 M PBS (pH 9.0) is added to the reaction until the pH is 8.0-8.5 and mixed. Then 100 µl of 0.25% glutaraldehyde (Type I - Sigma Chemical Co., St. Louis, Mo.) is added slowly with stirring for 10 minutes. The conjugate is then purified from reactants by gel filtration in phosphate buffered saline (PBS) and concentrated by ultrafiltration and washed at least twice in 2 volumes of PBS. Typically, 10 FAD/antibody are conjugated via this approach. Doxorubicin is reacted at 100 molar excess at 37°C for 60 minutes and continued at 4°C overnight. Free drug is removed from antibody-antimer-drug conjugate by a combination of gel filtration and ultrafiltration. Typically, one mole of drug is incorporated per FAD molecule conjugated to antibody.

A second example of using an antimer conjugation uses a dye, such as Reactive Blue 4 (RB4) (Aldrich Chemical Co.) coupled first to human serum albumin (HSA) and then conjugated to antibody. 2.6 mg of human serum albumin (at 10 mg/ml) at pH 8.5 in 0.2 M bicarbonate buffer is reacted with 7.5 mg of activated RB4 (alkylation of amino groups on protein by the chlorogroup on RB4. The RB4 at 20 mg/ml in H_2O is reacted for 1 hour at room temperature. 1.0 M Lysine at approximately pH 10 is used to quench the reaction. The conjugation is "cleaned up" by gel filtration, resulting in approximately 7 RB4 molecules/human serum albumin (HSA). The derivatized albumin is then reacted with 1 mg of SMCC in 10% DMSO/ethanol at pH 9.8 for 1 hour at room temperature. Excess reactants are removed by gel filtration. Antibody (10 mg) is reacted with 8 mg DTT at room temperature for 30 minutes. Excess reactants are removed by gel filtration in degassed PBS and antibody (SH) reacted with the SMCC derivitized albumin. The conjugate is then reacted with a 100 molar excess of doxorubicin overnight at 4°C, followed by the removal of the unbound drug.

Example 4

Assessment of Antimer/Drug Affinity

Affinity of binding of drug bound to soluble antimer or to antimer conjugated to antibody or carrier can be assessed for example, by competition with albumin or cardiolipin-containing liposomes. Cardiolipin is a strong acceptor of doxorubicin. Doxorubicin associated with liposomes is measured spectrophotometrically. Doxorubicin associated with albumin is also measured spectrophotometrically. Once albumin is separated from antibody by differential $(NH_4)_2 SO_4$ precipitation or binding to Cibracron Blue Sepharose, the two drug acceptors are used to challenge the drug bound to antibody or carrier and represent the dissociation rate in serum or at target all membranes. As an example, doxorubicin bound to the reduced form of flavin adenine dinucleotide on antibody or carrier shows no reduction in bound doxorubicin when challenged with albumin, but shows transfer of doxorubicin to cardiolipin liposomes. Reducing the number of bonds by using oxidized flavin adenine dinucleotide allows for more rapid transfer to cardiolipin-containing liposomes but still only a low level transfer to albumin. This system is used to test for the appropriate affinity of antimer-drug interaction and allows one to know, in advance, whether or not the number of non-covalent bonds in a drug/antimer complex results in an appropriate affinity for delivery and release of drug at tumor sites. In some cases where the acceptor is unknown, m mbranes prepared from appropriate target cells could be used as the competitor.

A second methodology allows on to approximate th cumulative non-covalent bond energy between antimer and drug. A number of current chemotherapeutic drugs are charged at physiologic pH. Doxorubicin is positively charged due to the amino group of the daunosamin sugar. When analyzed on an isoelectric-focusing agarose gel, doxorubicin migrates to the cathode and can be readily visualized. When complexed

with an antimer that stably and non-covalently binds doxorubicin and satisfies ionic interaction, the drug/antimer complex remains at the origin in an isolectric-focusing agarose gel. By adjusting the electrical field strength until dissociation of drug and antimer occurs, on indirectly tests th affinity of binding between antimer and drug.

Example 5

Potency and Selectivity Immunoconjugates of Drug/Antimer

Potency and selectivity of drug conjugates can be measured by in vitro cytotoxicity assays versus antigen-positive and antigen-negative cells. One such assay utilizes MTT dye uptake and metabolism to determine residual surviving cells.

Potency and selectivity of covalent and non-covalent antibody-antimer-drug conjugates of doxorubicin are illustrated in Figure 2. Two types of covalent conjugates are used as benchmarks. The first is a conjugate of doxorubicin that uses ECDI as the cross-linking agent. In the second case, doxorubicin is bound through the amino group of the daunosamine sugar to antibody via a cross-linking reaction with glutaraldehyde. The ECDI-bonded, non-metabolizable conjugate is the least potent of the three types of antibody conjugates on a mole/mole basis when compared to free drug on the same cell line. The amino-bonded conjugate gives higher potency values but shows no selectivity on an antigen-negative cell line. The antimer-drug immunoconjugate has higher potency than the free drug, and has better selectivity than the covalent conjugates. These results indicate that antimeric conjugation utilizing non-covalent interactions such as pi-pi ionic and hydrogen bonding of the drug to the antimer creates both a potent as well as a more selective conjugate.

Selectivity is improved, because the antimer occupies the functional groups of the drug and interacts with cell membranes in a non-antigen-specific manner. Upon binding of the antibody to cell surface antigens, the juxtaposition of the non-covalently bound drug with the lipid membrane of the cell is sufficient to cause the detachment of the doxorubicin and the transfer of the drug to acceptor sites within or upon the plasma membrane.

Example 6

Drug conjugation to partial or pre-existing antimers on a carrier. In this example, albumin which has both low affinity as well as fewer high affinity sites for doxorubicin is exposed to 100 moles of drug per mole of protein in the presence of a dehydrating agent as indicated in Table I. Without the dehydrating agent <2 moles of drug is bound/albumin and drug leaches rapidly. With the use of dehydrating agent 6-15 moles of drug are bound per mole of protein with both low and high affinity (e.g., rapid or slow leaching). The conjugate, after removal of excess free drug is lyophilized and reconstituted as a typical pharmaceutical and administered as a slow release form of doxorubicin.

The number of high affinity sites on albumin is increased by conjugation of an activated form of cardiolipin on AZP to albumin. Some of these "partial" antimers will be in juxtaposition to other functional groups of endogenous amino acids which will complete the antimeric structure. Conjugation of drug is carried out as above and results in drug/carrier conjugate with slower release properties.

Example 7

Pharmaceutical Preparations of Soluble Drug/Antimers

In most cases simple use of antimers, drugs such as doxorubicin are complexed with antimer either in

presence or absence of a dehydrating agent such as in Example 2. If a d hydrating agent is used, on compatible with intravenous administration like thylen glycol is preferable. As with the antimer, FAD, if conjugation to protein is not intended, additional functional group(s) are available to undergo further interaction with the drug producing higher affinity binding. Upon challeng with cardiolpin-liposomes only slow transfer kinetics are seen. These results, correlate with reduced cardiac toxicity, in vivo, of doxorubicin/antimer complex.

Example 8

Doxorubicin is covalently conjugated to NR-ML-05, a monoclonal antibody specific for the 250 kilodalton melanoma-associated antigen. The mechanism of covalent conjugation is well established in the field. Oxidized FAD, non-oxidized FAD, propanalol or other antimers for doxorubicin are added, and then dialyzed. The drug conjugate with non-covalently bound antimer is evaluated against antigen positive (M14+) and antigen negative (M14-) cell lines and is compared for selectivity in vitro by comparing IDSO values for the M14+ and M14- cells to free doxorubicin and to the doxorubicin-NR-ML-05 conjugate without antimer. The antimer is chosen that provides potency as close as possible to the conjugate not complexed with antimer, but also provides the most selectivity as defined by the in vitro assay. The antimer must bind to the drug or the antibody with sufficient stability, however, to remain complexed in human serum.

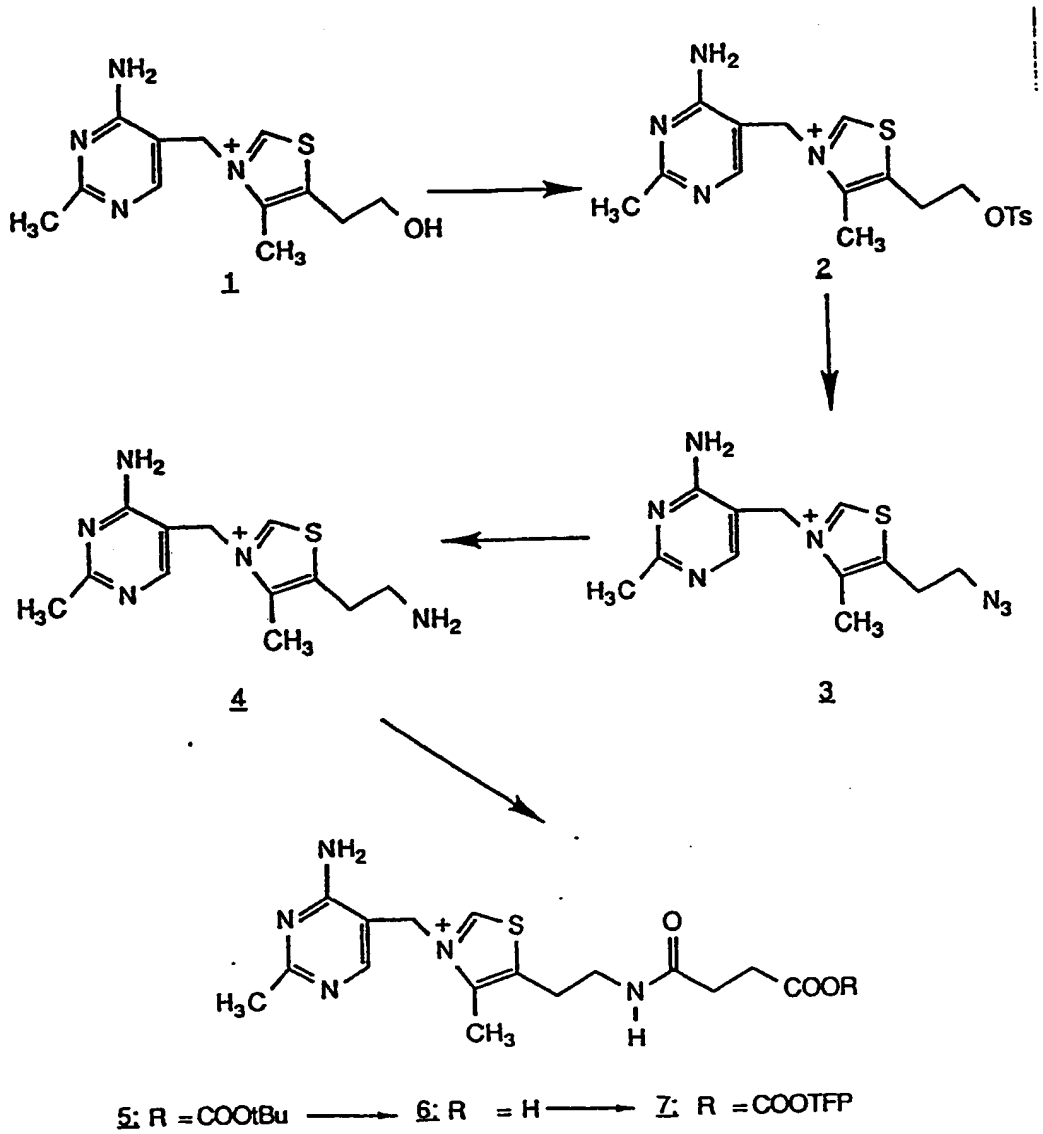
In another embodiment mitomycin-C is bound covalently to an antibody. FAD, oxidized FAD, propranalol or other antimers are added, and then the unbound antimer is dialyzed out. The conjugate of mitomycin-C is tested against antigen positive and antigen negative cells and compared to those conjugates that have been exposed to the different antimers. The procedure permits the selection of the most cytotoxic conjugate.

Example 9

Antimers to 5-Fluorouracil (5-FU)

An antimer to 5-FU is made by modifying the side chain of Thiamin (vitamin B_1) to make it susceptible to covalent binding to a targeting protein while retaining the intact functional groups on the pyrimidine ring so as to non-covalently bind through multiple interactions synthetic scheme to make compound 1, the 2,3,4,6-tetrafluorophenyl ester of 4-β-(N-γ-carboxy propionyl) aminomethyl-5-methyl-1-N-(R-methyl-4-aminopyrimidine-5-yl) methyl thiazole.

scheme 1



A solution of thiamin (compound 1 in scheme 1) is dissolved in 5 mL of anhydrous pyridine and cooled to 0°-5°C. p-Toluenesulfonyl chloride (1.1 mmole) is added in portions over a period of 5-10 minutes. The solution is stirred while refrigerated for 2-3 hours and can be stored overnight is kept refrigerated. Approximately 5-10 mL of water is added and the solution may be filtered, if necessary. If no precipitate forms, the solution is evaporated to dryness in vacuo. The dried residue is dissolved in ethyl acetate. The dissolved residue is washed with water and then dried over anhydrous sodium sulfate and evaporated to dryness to yield the product, O-p-toluenesulfonyl thiamin, depicted as compound 2 in Scheme 1.

Compound 2 is made into a 1 mmolar solution in 10 mL of anhydrous tetrahydrofuran. Trimethylsilyl azide (1.2 mmoles) is added to the solution and then the solution is refluxed for 6-8 hours. The solution is evaporated to dryness and then the remaining residue is dissolved in ethyl acetate. The new solution is washed with water and then dried over anhydrous sodium sulfate followed by evaporation to yield 4-β-Azidoethyl-1-N-(2-methyl-4-amino pyrimidin-5-yl)methyl thiazol shown as compound 3 in Schem 1. Compound 3 is purified by preparative liquid chromatography.

Compound 3 (1.0 mmole) in 10-15 mL of absolute ethanol, is hydrogenated at atmospheric pressure over sulfided Pd-C catalyst in a Paar apparatus. After hydrogenation, the catalyst is removed by filtration over celite and the solvent is removed in vacuo, leaving 4-β-Aminomethyl-1-N-(2-methyl-4-aminopyrimidinyl)methyl-5-methyl thiazole or Compound 4 in Scheme 1.

Compound 4 (1.0 mmole) is dissolved into 10 mL of anhydrous tetrahydrofuran and succinic acid mono-t-butyl ester mono succinimidate ester is added. The solution is mixed and then stirred for 2 hours at room temperature. The solvent is next evaporated. The residue is dissolved in methylene chloride and then washed with water. The organic layer is dried with anhydrous $MgSO_4$ and the evaporated to yield the derivative of compound 4 which is compound 5 in Scheme 1.

Compound 5 is converted to the free acid form by adding the t-butyl ester (1 mmole) to 10 mL of methylene chloride and 2 mL of anhydrous trifluoracetic acid and the solution is stirred for 30 minutes under refrigeration conditions. The solution is allowed to warm to room while the stirring is continued for another 3 hours. The solvents are removed in vacuo and the residue is coevaporated several times with the methylene chloride to ensure the complete removal of trifluoroacetic acid. Trituration with ether precipitates the free acid, which is compound 6 in Scheme 1. Compound 6 is purified by liquid chromatography.

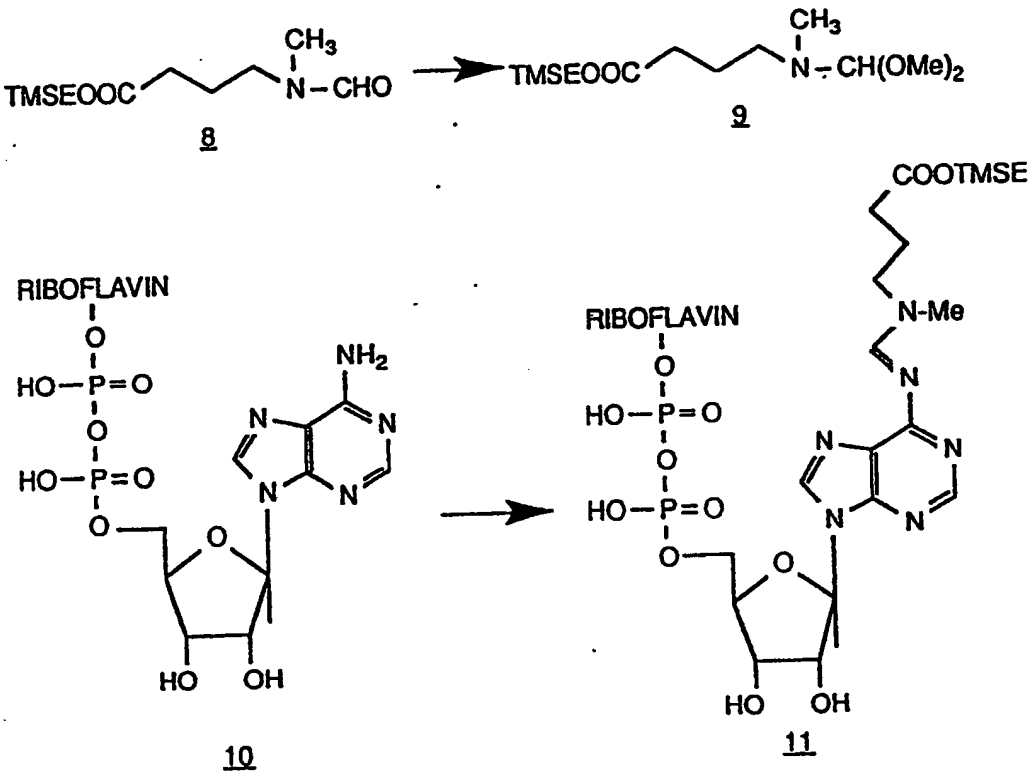
The 2,3,5,6-tetrafluorophenyl ester of the free acid is formed from a solution of 1 mmole of free acid (compound 6) dissolved in 10 mL of acetonitrile:water (4:1), 3 mL of tetrafluorophenol and 5 mmole of 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride and the resulting solution is stirred overnight at room temperature. The precipitated solid is filtered and then washed with ether to remove the free tetrafluorophenyl. The active ester is purified by high pressure liquid chromatography and is compound 7 in Scheme 1.

Example 10

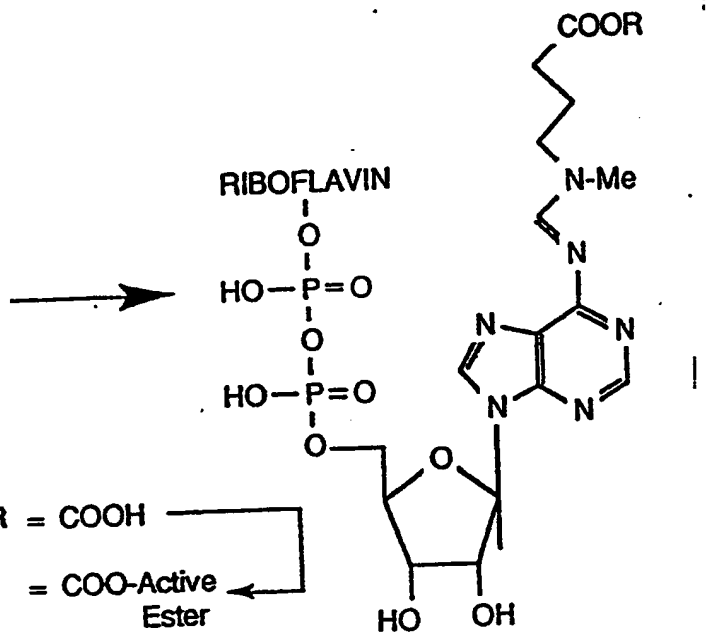
A modified FAD As A Doxorubicin Antimer

In this example, a modified FAD is made to become an antimer to doxorubicin. The resulting antimer, a riboflavin derivative contains antimeric binding cites for non-covalent interactions with doxorubicin and an active ester leaving gorup for covalent attachment to the targeting protein. The synthetic scheme for making the modified FAD antimer is illustrated in Scheme 2 below.

Scheme 2


TMSEOOC
CH3
N–CHO
8
TMSEOOC
CH3
N–CH(OMe)2
9
RIBOFLAVIN
HO–P=O
HO–P=O
NH2
HO
OH
10
COOTMSE
N-Me
RIBOFLAVIN
HO–P=O
HO–P=O
HO
OH
11

Scheme 2



Compound 8 in Scheme 2 is formed by mixing a solution of 2 mmole of 4-(methylamino)butyric acid in 10-15 mL of formic acid and 2-3 drops of acetic anhydride. The mixture is stirred for 30 minutes. The solvent is evaporated and titrated with ether to yield the product of the reaction, N-formyl-4-(methylamino)-butyric acid, which in the quantity of 1 mmole is added to anhydrous tetrahydrofuran and stirred with 1.1 mmole of trimethylsilyethanol (TMSE) from Aldrich Chemical, and 1.1 mmole of N,N'-dicyclohexylcarbodiimide for an overnight time period. A precipitated solid will be formed. The solid is filtered and the filtrate is evaporated to dryness. The residue is dissolved in ethyl acetate and washed with water. The organic layer is dried and evaporated to give N-formyl-4-(methylamino)butyric acid trimethylsilylethyl ester, and the ester (compound 8) is refluxed with methanol (20 mL/mmole of the compound) containing a catalytic amount of p-toluenesulfonic acid for 5-6 hours. The methanol is completely removed in vacuo and compound 9 in Scheme 2 is purified by column chromatography.

FAD (flavine adenine dinucleotide, Pierce Chemical; compound 10) 1 mmole is dissolved in 20 mL of acetonitrile:water (1:1) and compound 9 of scheme 2, N-dimethoxymethyl-N-methyl-4-(methylamino)butyric acid trimethylsilyl ethyl ester (2-3 mmole) is added and the mixture is stirred for 6-10 hours at room temperature. The solvents are removed in vacuo and the residue is suspended in water and extracted with ethyl acetate to remove the excess of compound 9. The product, flavin N^6-(methyl,γ-carboxy-propyl) aminomethylene adenosyl dinucleotide, compound 11 in scheme 2 is isolated from the aqueous solution by freeze-drying followed by liquid chromatography purification.

A 1 mmole concentration of compound 11 is dissolved in 2 mL of triethylammonium bicarbonate and then evaporated to dryness. Approximately 5 mL of water is added to the residue and 2 mmole of potassium fluoride or tetraethyl ammonium fluoride is also added and the mixture is stirred for approximately 30 minutes. The mixture is evaporated to dryness. Acetone is added and then the solution is coevaporated. The residue is dissolved in 1:1 ethanol-acetone mixture to which a saturated solution of $NaClO_4$ is added. A solid is precipitated and isolated by centrifugation and then dried in a vacuum desicator and purified by liquid chromatography by using isopropanol-acetic acid-water as mobile phase.

One mmole of the free acid compound 12 is added to 5 mL f acetonitrile:water (1:1) and 3 mmole of 2,3,5,6-tetrafluorophenol and 3 mmole of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride is added to the solution and the solution is stirred for 10-12 hours at room temperature. The solution is diluted and extracted with ether to remove excess tetrafluorophenol and the product, flavin N^6-(methyl-2,3,4,5,6-

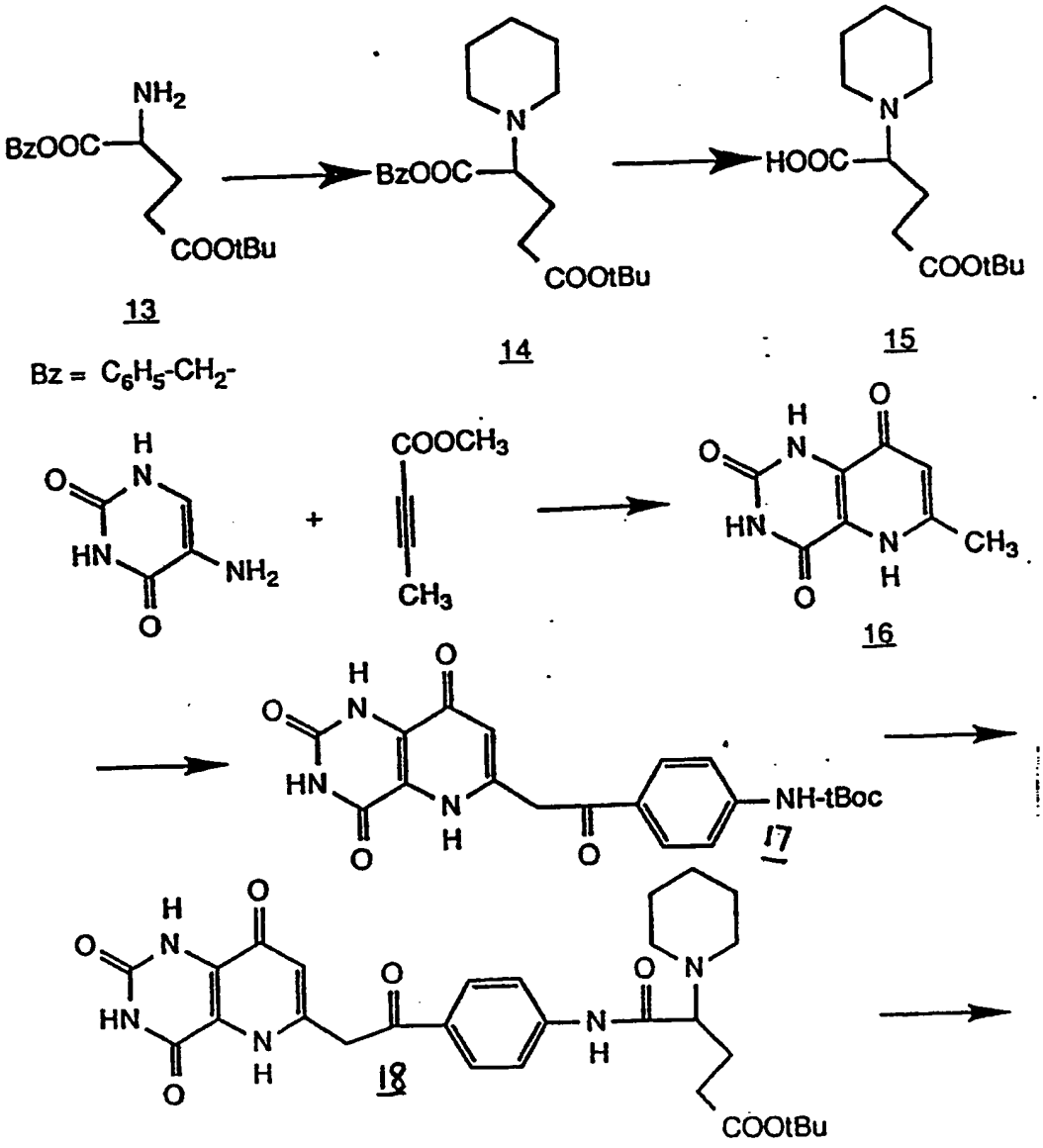
tetrafluorophenoxycarbonylpropyl) aminomethylene-adenosyl dinucleotide, compound 13 in schem 2, is isolated by high pressure liquid chromatography.

Example 11

Antimer for Methotrexate

An antimer to methotrexate (MTX) can be formed that utilizes both ionic interactions and hydrogen bonding interactions to non-covalently bind MTX to the antimer. Methotrexate will non-covalently bind to the antimer via its pteridine ring to the pyridophyrimidine ring of the antimer and with the pyrolidine ring of MTX to the α carboxylic acid of the glutamic acid portion of MTX. Scheme 3 below illustrates the synthetic procedure necessary to form the antimer α-1-pipperdinyl-α-N-p-(2,4,8 trioxopyrido [3,2-d] pyrimidin-6-yl) acetylphenyl amido glutaric acid-γ-succinimidate ester.

SCHEME 3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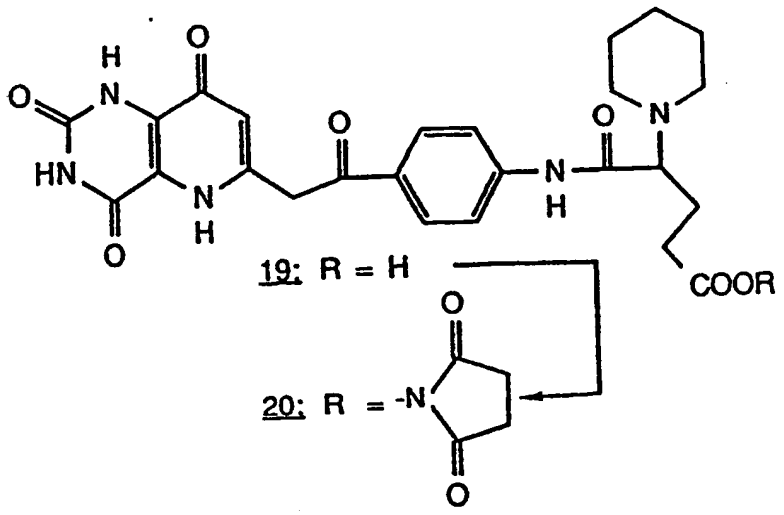
BzOOC
COOtBu
13
Bz = C6H5-CH2-
BzOOC
COOtBu
14
HOOC
COOtBu
15
COOCH3
CH3
16
NH-tBoc
17
18
COOtBu

Scheme 3



A 5 mmole concentration of compound 13, glutamic acid-α-benzyl ester-γ-t-butyl ester is added to 5 mL of a 25% solution of glutaraldehyde and the solution is stirred for 2-3 hours at room temperature. To this solution is added 10 mmoles of sodium cyanoborohydride and the stirring is continued for another 2 hours. The aqueous phase of the solution is evaporated and the residue is suspended in water and extracted with ethyl acetate. The organic layer is washed with water, dried over anhydrous sodium sulfate and then evaporated to give compound 14. Compound 14, 2-(pyrrolidin-1-yl)-glutaric acid-1-benzyl ester-5-t-butyl ester is purified by prepared liquid chromatography.

A solution of 2 mmole of compound 14 is prepared in 20 mL of methanol containing two equivalents of hydrogen chloride and hydrogenated in a Paar apparatus over Pd-C (10%) for 3 hours. The catalyst is filtered throug celite and the filtrate is evaporated to give the product, compound 15, as a hydrochloride salt.

To a suspension of 10 mmole of 5-aminouarcil in 100 mL of methanol is added 12.5 mmole of methyl-2-butynoate and the suspension is stirred at room temperature for 8 hours. The precipitated solid is filtered and dried in vacuo to give 4-(2',4'-dioxopyrimidin-5-yl) amino crotonate. A suspension of the crotonate is refluxed for 3 hours. The mixture is cooled to room temperature and petroleum ether is added. The precipitated solid is filtered and air dried to give compound 16, 2,4,8-trioxo-6-methylpyrido[3,2-d] pyrimidine, which is purified by crystallization.

To a 2 mmole solution of p-aminobenzonitrile in 10 mL of dry dimethylformamide, 3 mmole of di-t-butyl dicarbonate is added and the solution is stirred for 3-5 hours at room temperature. The solvent is removed in vacuo and the residue is suspended in water and extracted with methylene chloride. The organic layer is washed with water, dried with anhydrous $MgSO_4$ and evaporated to give p-N-t-butoxycarbonylaminobenzonitrile, which is purified by crytallization. A 5 mmole suspension of compound 16 is refluxed with 5 mL of hexamethyldisilazane and 1 mL of chlorotrimethylsilane and 50 mL toluene. After a clear solution is obtained, the solvent is removed with the exclusion of moisture to obtain the trimethylsilyl derivative of compound 16. This derrivative is dissolved in 10 mL of anhydrous tetrahydrofuran (THF). The trimethylsilyl derivative of compound 16 in solution is added to a cooled solution to 6 mL of 1 molar n-butyllithium in anhydrous THF. A red solution is obtained indicating the formation of α-methyllithium derivative of compound 16. After approximately 30 minutes of stirring, 5 mmole of p-N-t- butxoycarbonylaminobenzonitrile in 5 mL of anhydrous THF is added in drops over a period of 5 minutes. After another 10-15 minutes of stirring, 10 mL of 1 N hydrochloric acid is added to hydrolyze the amine formed in the reaction. The solvents are removed in vacuo and the residue is crystallized to give the product, 2,4,8-trioxopyrido [3,2-d] pyrimidin-6-yl) methyl-(p-t-butoxycarbonyl)-amino phyenyl keton , compound 17.

Two mmole of compound 17 is dissolved in approximately 10 mL of m thylene chloride and 2 mL of trifluoroacetic acid and stirred for 3 hours. The solvents are removed and the residue is titrated with ether to yield an aniline intermediate. A 2 mmole solution of the aniline intermediate is stirred with 2 mmol of

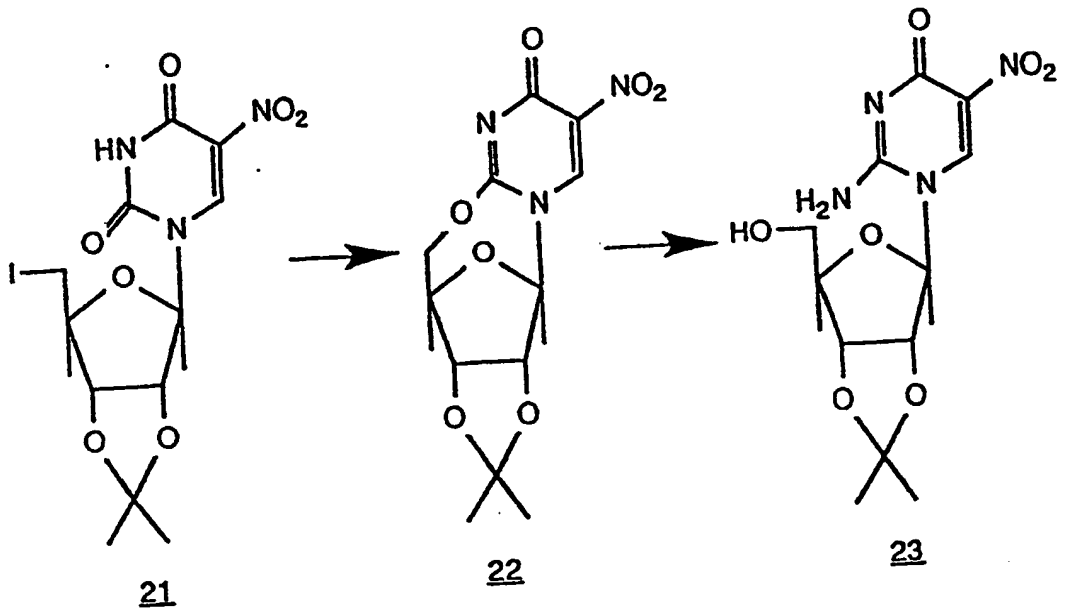
compound 15 above in 20 mL of acetonitrile:water (1:1) and 5 mmole of 1-(dimethylaminopropyl)-3-ethylcarbodiimide. After the reaction is completed the solvents are removed and the product, compound 18, α-1-pipperdinyl-α-N-p-(2,4,8- trioxopyribo [3,2-d] pyrimidin-6-yl) acetylphenyl amido glutaric acid-γ-t-butyl ester is isolated by silica gel chromatography. Two mmol is compound 18 is stirred with 10 mL of methylene chloride and 2 mL of trifluoroacetic acid for 3 hours. The solvents are removed and the residue is triturated with ether to give the acid intermediate to prepare the active ester. The acid intermediate is compound 19 in scheme 3. A solution of compound 19 (1 mmole) in anhydrous acetonitrile is stirred with 1.1 mmole of N-hydroxisuccinimide and 1.1 mmole of N,N'-dicycloatxylcarbodiimide for 10-12 hours at room temperature. The precipitated dicyclohexylurea is filtered and the filtrate is evaporated to dryness. The residue is dissolved in ethylacetate and then washed with water. The organic layer is dried with anhydrous sodium sulfate and evaporated in vacuo to give the product an antimer depicted in scheme 3 as compound 20, which is α-1-pipperdinyl-α-N-p-(2,4,8-trioxopyrido [3,2-d] pyrimidin-6-yl)-acetylphenyl amido glutaric acid-γ-succinimidate ester. The final purification is carried out by high pressure liquid chromatography.

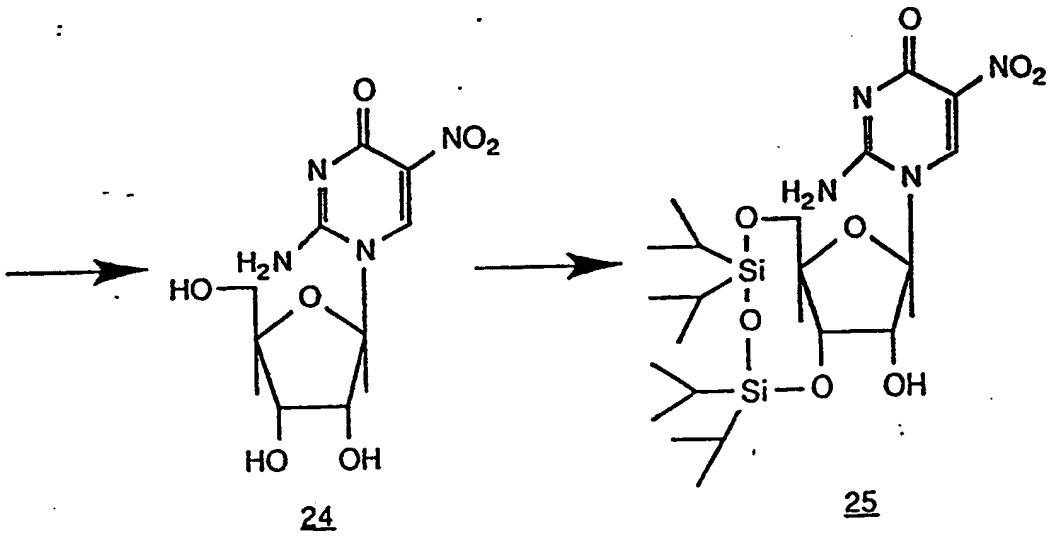
Example 12

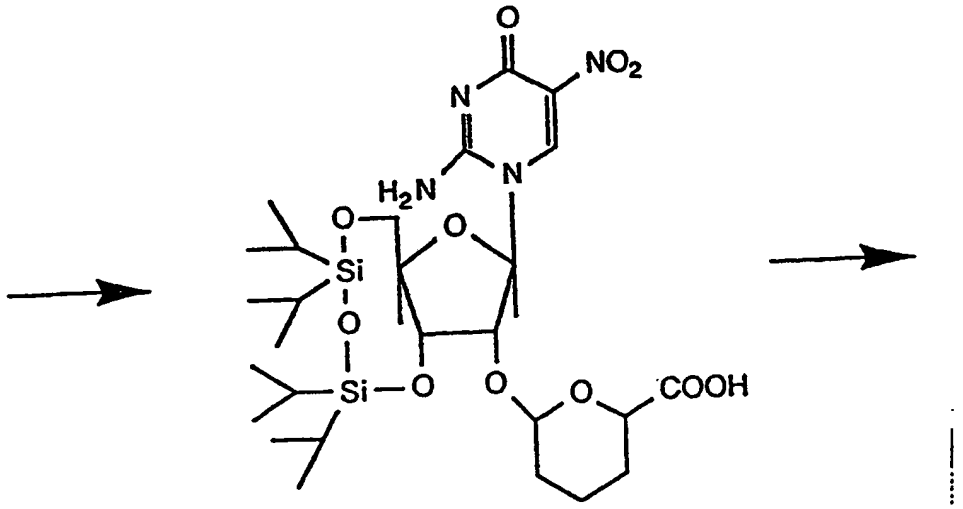
An Antimer for Cytosine Arabinoside (ARA-C)

An antimer for ARA-C is prepared by designing a molecule which reacts with the nitroisocytidine groups of the ARA-C molecule by ionic interactions and hydrogen bonding. The rest of the antimer molecule is designed to confer aqueous soluability to the antimer. The preparation of the antimer to ARA-C is described in scheme 4.

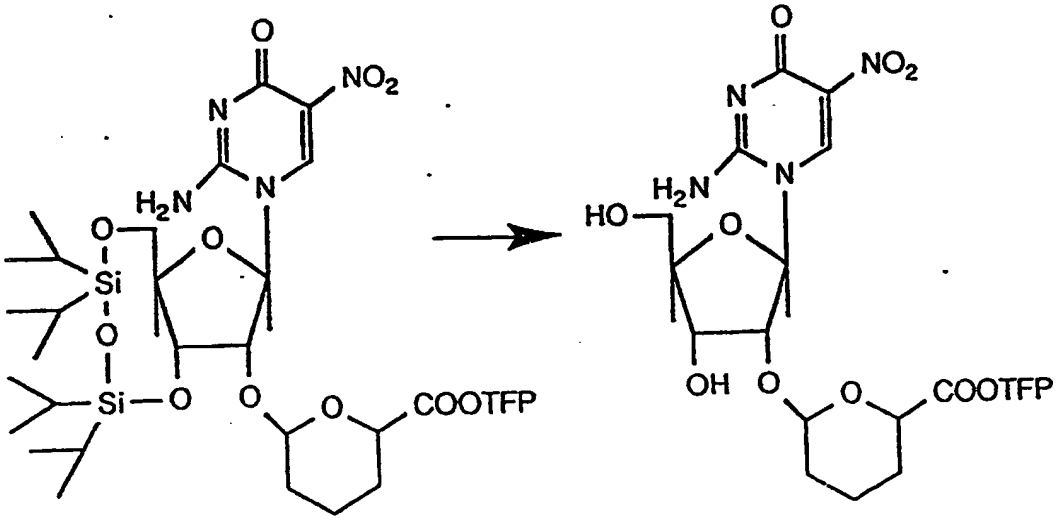
Scheme 4







26



27 28

Compound 21 of scheme 4, 5′-deoxy-5-iodo-2′,3′-0-isopropylidene-5-nitrouridine is prepared by the procedure similar to the one used by Brown et al., J. Chem Soc., 868 (1957), except the starting material is 5-nitrouridine instead of uridine. Compound 21 is converted to 2,5′-anhydro-2′-3′-0-isopropylidene-5-nitrouridine which is compound 22 in scheme 4. Compound 22 is reacted with saturated methanolic ammonia to give 2′,3′-0-isopropylidene-nitroisocytidine, which is compound 23. Compound 23 is deprotected using 98% formic acid to give 5-nitroisocytidine, which is compound 24.

A solution of 2 mmole of compound 24 is prepared with 20 mL of anhydrous tetrahydrofuran and containing 2.5 mmole of triethylamine and 1.1 mmole of 1,3-dichloro- 1,1,3,3-tetraisopropyldisiloxane in 2 mL of tetrahydrofuran. The solution is stirred for 8 hours and evaporated to dryness. The residue is dissolved in m thylene chloride and purified by flash chromatography over sicila gel. The r sidue is compound 25, which is 3′,5′-tetraisopropyl-disiloxy-5-nitroisocytidine.

A solution of 1 mmole of compound 25 is prepared in 10 mL of anhydrous THF containing a catalytic amount of P-tolunesulfonic acid, and 1.1 mmole of 3,4-dihydro-2H-pyran-2-carboxylic acid. The solution is stirred for 3-4 hours and then evaporated in vacuo to yield compound 26 which is 6″-carboxy-2′-tetrahydropyran-2″-yl-3′,5′-tetra-isopropyl-disiloxy-5-nitroisocytidine. Compound 26 is purified by column chromatography. The tetrafluorophenyl ester of compound 26 is prepared by the same procedure as described in Exampl 10. The tetrafluorophenyl ester of compound 26 is shown in scheme 4 as compound 27

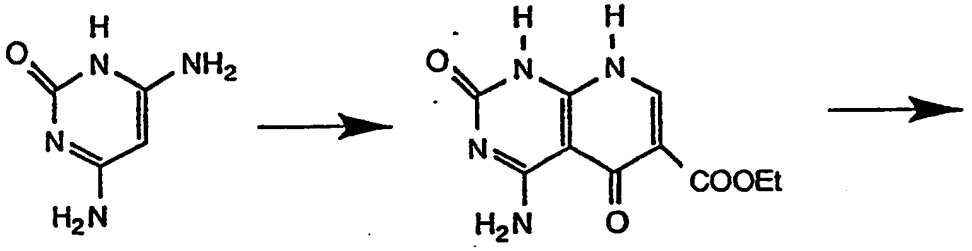
One mmole of compound 27 is mixed with 1.1 mL tetra-n-butyl-n-ammonium fluoride in THF with 10% water for 30 minutes. The solvent is removed in vacuo and the residue containing the product, compound 28, 6″-(2,3,5,6-tetrafluorophenoxycarbonyl)-2′-tetrahydropyran-2″-yl-5- nitroisocytidine, compound 28, is purified by silica gel flash chromatography and a final purification by high pressure liquid chromatography.

Example 13

An Antimer for Mitomycin C

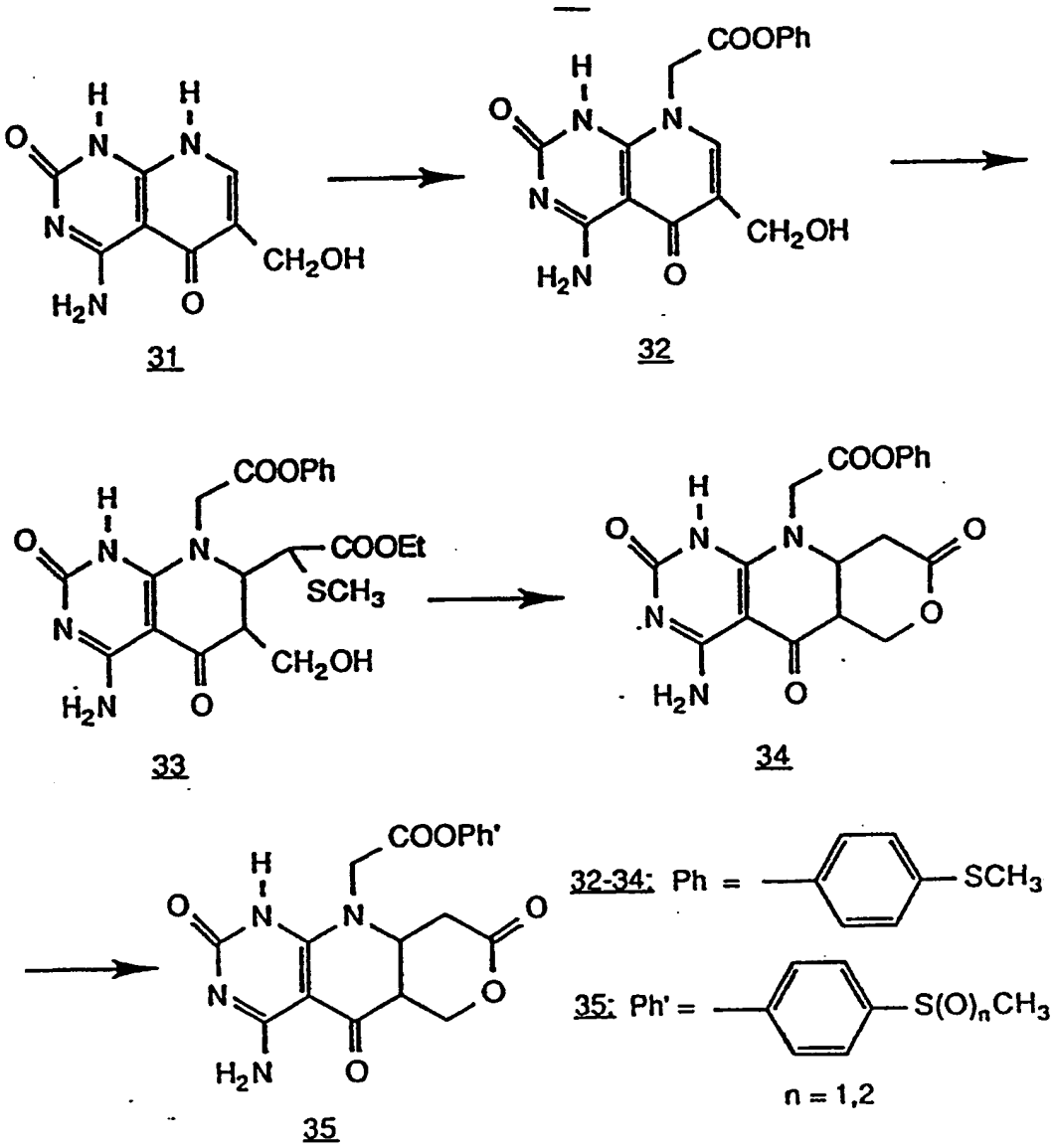
Scheme 5 depicts the synthetic procedure to form the active ester antimer for mitomycin C.

Scheme 5



29 30

Scheme 5



A mixture of 5 mmole of 4,6-diamino-2-oxopyrimidine and 10-15 mmole of diethylethoxymethylene malonate is mixed and heated at 120° to 150° C for 6-8 hours. A clear melt is obtained. The temperature is maintained to facilitate the removal of ethanol. After the ethanol is removed, the mixed is allowed to cool to room temperature and the solid mass is broken up with ethanol and filtered to give the intermediate product, 2-(4-amino-2- oxopyrimidin-6-yl) aminomethylene malonate. The above malonate derivative is stirred and heated in Dowtherm A for 3-5 hours to complete the cyclization yielding product 30 in scheme 5, 4-amino-6-carbethoxy-2,5-dioxopyrido [2,3-d] pyrimidine. The precipitated solid is filtered, washed with petroleum ether and recrystallized prior to the reduction step.

A suspension of compound 30 in 20 mL of anhydrous THF is made in 2 mmole of $LiAlH_4$ is added in small portions. The progress if the reduction is monitored by TLC (thin later chromatography). If the reduction is incomplete, portions of the $LiAlH_4$ is added to complete the reaction. A small quantity of ice-cold water is added in drops to d stroy the excess reagent and then is filtered out. The filtrate is acidified with glacial acetic acid and evaporated to give th product, compound 31, 4-amino-6-hydroxymethyl-2,5-

dioxopyrido [2,3-d] pyrimidine. Compound 31 is recrystallized.

A solution of 1 mmol of compound 31 in anhydrous dimethylformamide, containing 2 mmole of anhydrous potassium carbonate and chloroacetic acid p-(methylthio) phenyl ester (1 mmole); prepared by DCC mediated condensation of chloroacetic acid with p-(m thylthio phenyl by standard procedures) is added and the mixture is stirred overnight. The solvent is removed in vacuo and the residue is suspended in water, acidified, filtered and crystallized to yield the product compound 32, 4-amino-6-hydroxymethyl-2,5-dioxopyrido [2,3-d] pyrimidine-N^8-acetic acid p-methylthio) phenyl ester.

A solution of the sodium salt of methylthioacetic acid ethyl ester in tetrahydrofuran is prepared by the addition of sodium hydride to methylthioacetic acid ethyl ester. This solution is added to a solution of compound 32 in anhydrous dimethylformamide (DMF) and the solution is heated to 50° to 60° C to complete the reaction. The progress of the reaction is monitored withdrawing samples, acidifying with acetic acid to quench the reaction and observing the progress of the reaction by TLC. When the reaction is judged complete, the solution is acidified with acetic acid and the product is obtained by evaporation of the solvent followed by aqueous work-up to yield compound 33, 4-amino-6-hydroxymethyl-2,5-dioxopyrido [2,3-d] pyrimidine-7-(α-methylthio) acetic acid ethyl ester-N^8-acetic acid p-(methylthio) phenyl ester.

One mmole of compound 33 is briefly heated in ethanol with 1 gram (wet weight) of Raney Nickel to dethiate the 7-α-thiomethyl group. After the completion of the reaction, 1 mL of 1 N HCl is added to the solution and the heating is continued to complete the lactonization. The product is obtained by evaporation of the solvents in vacuo, followed by crystallization, to yield the lactone derivative, which is compound 34. A suspension prepared with 1 mmole of the lactone derivative in 10 mL of anhydrous tetrahydrofuran and stirred with two equivalents of m-chloroperoxybenzoic acid for 3-5 hours. This reaction yield a mixture of sulfone and sulfoxide derivatives. The products are obtained by filtration.

Example 14

Oligopeptides Comprising Side Chains for Intercalation of Doxorubicin

Experiments have been conducted to assess the use of synthetic oligopeptides to bind the drug doxorubicin. Binding was followed by changes in the visible spectrum of doxorubicin, and analyzed by titrating the drug absorption spectrum at 475 nm or 565 nm with varying concentrations of peptide, in pH 7.0 0.1 M phosphate buffer. Binding curves were fit to a single hyperbola with the program Enzfitter (Elsevier-Biosoft). Results with oligopeptides used to screen for binding are shown in Table 2. These peptides were designed to test the effectiveness in intercalating doxorubicin of a 1,3-spacing between amino acids containing aromatic groups in their side chains (i.e., a single non-aromatic amino acid intervenes between the two residues having aromatic side chains). The amino acids are C, cys; K, lys, W, trp; G, gly; E, glu; D, asp; Fmoc, 9-fluorenylmethoxycarbonyl, attached to the lysine epsilon-amino group; and MIANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid attached to cysteine.

The oligopeptides were synthesized using the boc-benzyl solid-phase peptide synthesis strategy of Barany and Merrifield as described in The Peptides: Analysis, Synthesis, Biology, E. Gross and J. Meienhofer (eds.), New York: Academic Press (1980).

TABLE 2

Binding of Doxorubicin by Synthetic Oligopeptid s	
Peptide Sequence	Kd, μM
CKWGWK-amide	no detectable binding
CKWGWGWK-amide	same
CKWGWKWGWK-amide	same
KK(Fmoc)GK(Fmoc)KGGC	same
EK(Fmoc)GGK(Fmoc)EGGC	same
EK(Fmoc)K(Fmoc)EGGC	same
EK(Fmoc)GK(Fmoc)EGGC	280
DK(Fmoc)GK(Fmoc)DGGC	22
EK(Fmoc)EK(Fmoc)EGGC	no detectable binding
EEK(Fmoc)GK(Fmoc)EEGGC	33
EC(MIANS)GC(MIANS)EGGC(Acm)	48

Figure 3a is presented as an example of a titration curve resulting from these experiments, and shows the best-fit curve to titration of 50 μM doxorubicin with varying concentrations of the oligopeptide DK-(Fmoc)GK(Fmoc)DGGC-amide. No absorbance change was observed when the oligopeptide was added to buffer in the absence of doxorubicin. A concentration-dependent, saturable increase in absorbance occurs when oligopeptide is added to drug, suggesting formation of a complex betwen the two components. The oligopeptide carrier DK(Fmoc)GK(Fmoc)DGGC-amide was added in varying concentrations to doxorubicin (50 μM in 0.1 M phosphate buffer at pH 7.0), and changes in the absorption spectrum of doxorubicin were observed at 475 nm. Oligopeptide carrier alone was added to the reference cuvette. The binding constant is derived from the nonlinear least squares best fit to a single hyperbola using the program Enzfitter (Elsevier-Biosoft), yielding an apparent dissociation constant of 22 μM.

Figure 3a shows the results for titration of doxorubicin with the oligopeptide EC(MIANS)GC(MIANS)-EGGC(Acm). This oligopeptide was prepared by first synthesizing the oligopeptide ECGCEGGC(Acm), wherein "Acm" represents an acetamidomethyl protecting group. The synthesis was conducted using the solid phase methodology of Barany and Merrifield supra and Houghten, R.A. (1985), Proc. Nat. Acad. Sci. USA 82, 5131. The number of cysteine thiols per peptide was 1.8 as measured by titration with Ellman's reagent. See Means, G.E.; Feeney, R.E. (1971), Chemical Modification of Proteins, San Francisco: Holden-Day, p. 220.

The peptide concentration was determined with ninhydrin. (See Scheraga, H, (1978), Pure Appl. Chem. 50, 315.) The oligopeptide then was alkylated with two moles of MIANS per mole of oligopeptide. MIANS (Molecular Probes, Eugene, Ore.) was derivatized to the two cysteine thiols of the peptide, and binding was followed as in Figure 1a in pH 7.0 0.1 M phosphate buffer, at 565 or 580 nm. The best-fit apparent dissociation constant was 48 μM.

The data suggest that aromatic side chains in a 1,3 spacing (as opposed to 1,2 or 1,4 spacing) can help bind doxorubicin. Including amino acids with negatively charged side chains (e.g., aspartate or glutamate) in the oligopeptide, and the position therein, also appears to be important for drug binding. This may be due to ion pair formation between the positively charged amino group on the daunosamine moiety of doxorubicin and either glutamate or aspartate.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustrations, various modifications may be made without deviating from the spirit and scope of the invention.

The features disclosed in the foregoing description, in the claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

Claims

1. A targeting protein/antimer/drug complex comprising:
a targ ting prot in;
a drug moiety; and
an antimer moiety non-covalently bound to said drug moiety, and coval ntly bound to said targeting protein.

2. The complex of claim 1 wherein the targeting protein is a monoclonal antibody or monoclonal antibody fragment.

3. The complex of claim 1 wherein the drug moiety has antineoplastic activity or is cytotoxic to tumor cells.

4. The complex of claim 1 wherein the antimer moiety is bound to the drug moiety via interactions selected from the group consisting of ionic, hydrogen, pi-pi, hydrophobic interactions, van der Waal forces, and combinations thereof.

5. The complex of claim 1 wherein the drug moiety comprises a planar ring structure and the antimer moiety comprises a planar ring structure that non-covalently binds to the planar ring structure of the drug moiety.

6. The complex of claim 1 wherein the drug moiety is selected from the group consisting of doxorubicin and other anthracycline derivatives, actinomycin D, ellipticine, and mitomycin-C.

7. The complex of claim 1 wherein the antimer moiety has a chemically opposing functional group to one or a plurality of the functional groups on the drug moiety.

8. The complex of claim 7 wherein the antimer moiety has an electron-poor functional group to sterically correspond to each electron-rich functional group on the planar ring structure of the drug moiety, and/or wherein the antimer moiety has an electron-rich functional group to sterically correspond to each electron-poor functional group on the planar ring structure of the drug moiety.

9. The complex of claim 7 wherein the antimer moiety has a hydrogen-bonding negative dipole to sterically correspond to a positive dipole on the drug moiety and/or the antimer moiety has a hydrogen-bonding positive dipole to sterically correspond to a negative dipole on the drug moiety.

10. The complex of claim 7 wherein the antimer moiety non-covalently binds to the drug moiety with at least one hydrophobic and/or pi-pi interaction.

11. The complex of claim 1 wherein the drug moiety is doxorubicin and the antimer moiety is flavin adenine dinucleotide or a derivative thereof.

12. The complex of claim 1 wherein the antimer is Reactive Blue 4.

13. A targeting protein/drug/antimer complex comprising:
a targeting protein;
a drug moiety joined to the targeting protein directly or by a linking group; and
an antimer moiety non-covalently bound to said drug moiety, whereby the antimer serves to protect functional groups on the drug moiety and to reduce non-specific interactions between the drug moiety and non-target cells.

14. The complex of claim 13 wherein the antimer moiety and the drug moiety have planar fused ring structures and the complex further comprises a plurality of non-covalent interactions between the antimer moiety and the drug moiety, wherein the planar fused ring antimer moiety has an opposing functional group to one or a plurality of functional groups on the planar fused ring drug moiety.

15. A targeting protein/carrier/drug/antimer complex comprising:
a targeting protein;
a carrier bound to either a carbohydrate or amino acid residue on the targeting protein;
a drug moiety bound to said carrier; and
an antimer moiety non-covalently bound to said drug moiety, whereby the antimer serves to protect functional groups on the drug moiety and to reduce non-specific interactions between the drug moiety and non-target cells.

16. A method of joining an antimer to a drug with a plurality of non-covalent bonds comprising mixing the drug and the antimer with a dehydrating agent.

17. The method of claim 16 wherein the dehydrating agent is selected from the group consisting of glycerol, propylene glycol, ethylene glycol, sodium sulfate and ammonium sulfate.

18. An antimer/drug complex for controlling the bioavailability and toxicity of the active drug moiety, comprising a drug molecule non-covalently bound to an antimer molecule with a plurality of non-covalent interactions.

19. The complex of claim 18 wherein the serum half-life of the drug molecule is increased by at least 10%.

20. An antimer that non-covalently binds to 5-Fluorouracil consisting essentially of a thiamin derivativ .

21. The antimer of claim 20 wherein the thiamin derivativ is selected from the group consisting of compounds 5, 6, and 7, as shown in scheme 1.

2. An antimer than non-covalently binds to doxorubicin consisting essentially of a modified flavin adenine dinucleotide.

23. The antimer of claim 22 wherein the modified flavin adenine dinucleotide is the free acid or active ester of Flavin N^5(methyl γ-carboxy-propyl) amino methylene adenosyl dinucleotide.

24. An antimer that non-covalently binds to methotrexate consisting essentially of the free acid or succinimidate ester of α-1-pipperdinyl-α-N-p-(2,4,8-trioxopyrido [3,2-d] pyrimidin-6-yl) acetylphenyl amido glutaric acid.

25. An antimer that non-covalently binds to cytosine arabinocide consisting essentially of the free acid or active ester of 6"-Carboxy-2'-tetrahydropyron-2"-yl-5-nitroisocytidine.

26. An antimer that non-covalently binds to mitomycin C consisting essentially of the sulfone or sulfoxide derivatives of 4-amion-6-hydroxymethyl-2,5-dioxopyrido [2,3-d] pyrimidine-7-(α-methylthio)acetic acid ethyl ester-N^8-acetic acid p-(methylthio)phenyl ester.

27. An antimer for the drug doxorubicin selected from the group consisting of oxidized flavin adenine dinucleotide, non-oxidized flavin adenine dinucleotide and propranol.

28. A complex comprising an aromatic drug non-covalently bound to an oligopeptide comprising at least two aromatic side chains.

29. A conjugate comprising an aromatic drug non-covalently bound to an oligopeptide comprising at least two aromatic side chains, and a targeting protein attached to the oligopeptide.

30. The conjugate of claim 29 wherein the oligopeptide comprises at least two naturally occurring amino acids that have aromatic side chains.

31. The conjugate of claim 29 wherein aromatic compounds that are not part of naturally occurring amino acids are attached to the oligopeptide as the aromatic side chains.

32. The conjugate of claim 29 wherein the drug is bound by intercalation between two of the aromatic side chains.

33. The conjugate of claim 29 wherein the oligopeptide additionally comprises at least one amino acid selected from the aspartic acid and glutamic acid.

34. The conjugate of claim 29 wherein a single non-aromatic amino acid intervenes between two amino acids bearing the aromatic side chains in the oligopeptide.

35. The conjugate of claim 29 wherein the drug is doxorubicin.

36. The conjugate of claim 29 wherein the aromatic drug is an anticancer drug and the targeting protein is a monoclonal antibody that binds to cancer cells.

37. The conjugate of claim 29 wherein the aromatic drug and each aromatic side chain comprise multiple aromatic rings.

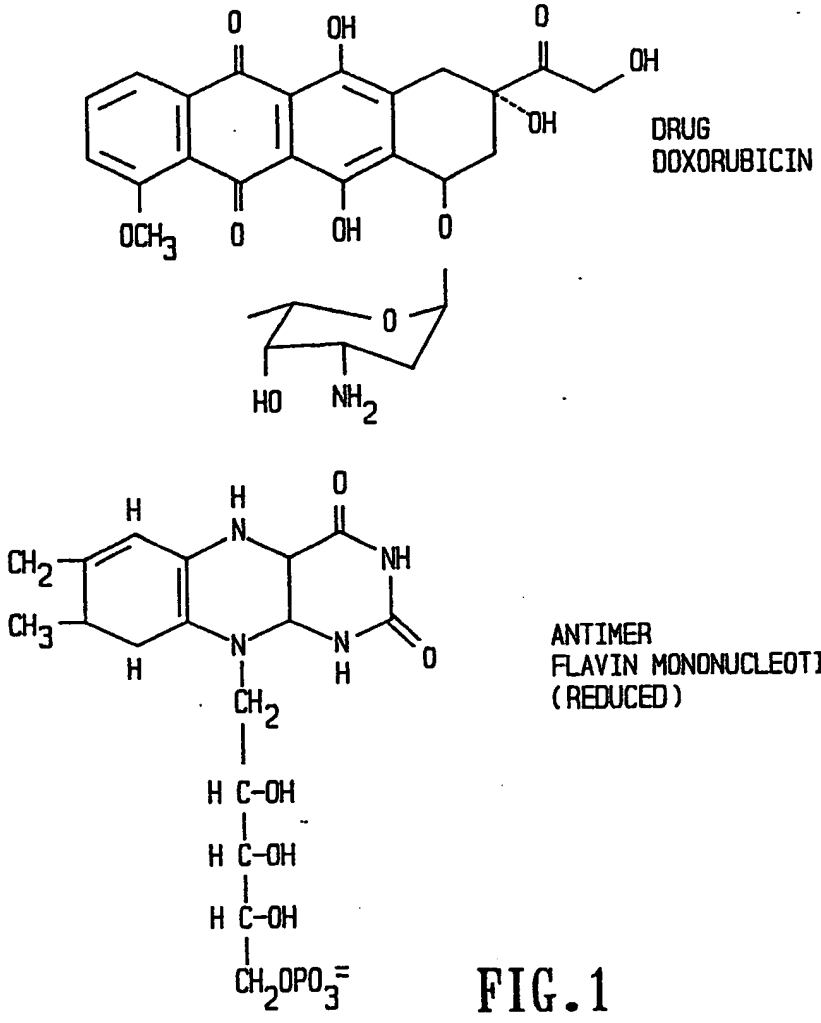
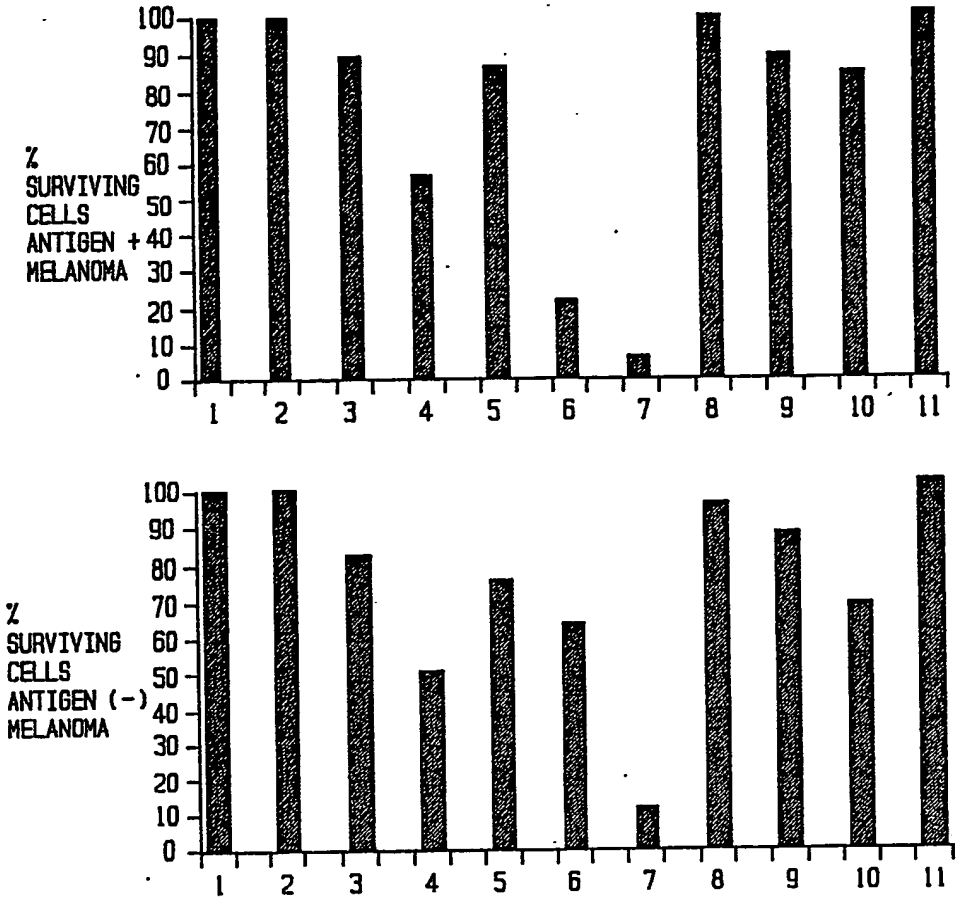


FIG.1



1 NrML-05 alone
2 Dox 10 ng/ml
3 " 50 ng/ml
4 " 100 ng/ml
5 FAD/NrML-5/Dox 10 ng/ml Dox
6 " 50 ng/ml "
7 " 100 ng/ml "
8 Dox/NrML-5/FAD e 10 NG/ML Dox
9 " 50 ng/ml "
10 " 100 ng/ml "
11 FAD/NrML-5 alone

FIG.2

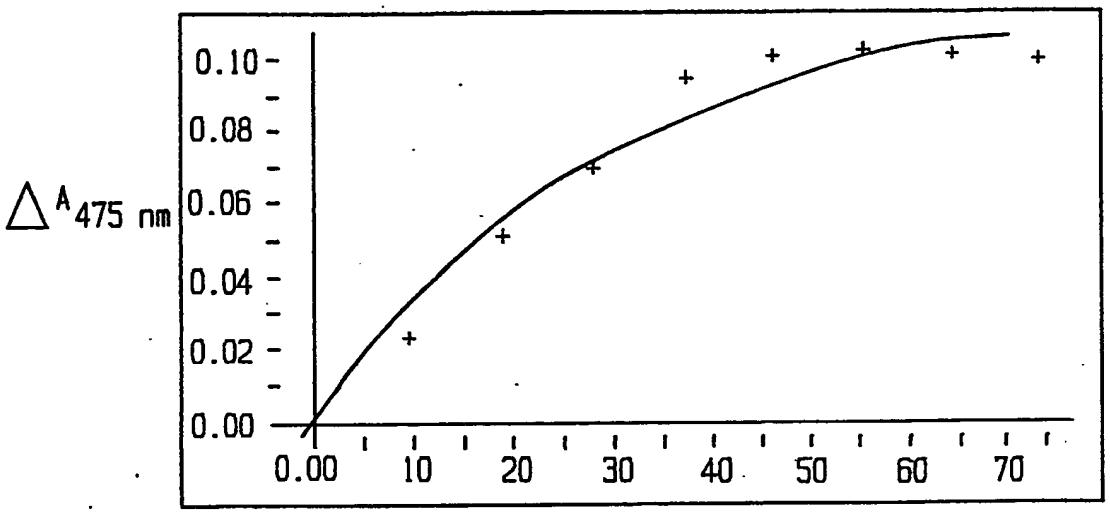


FIG.3A

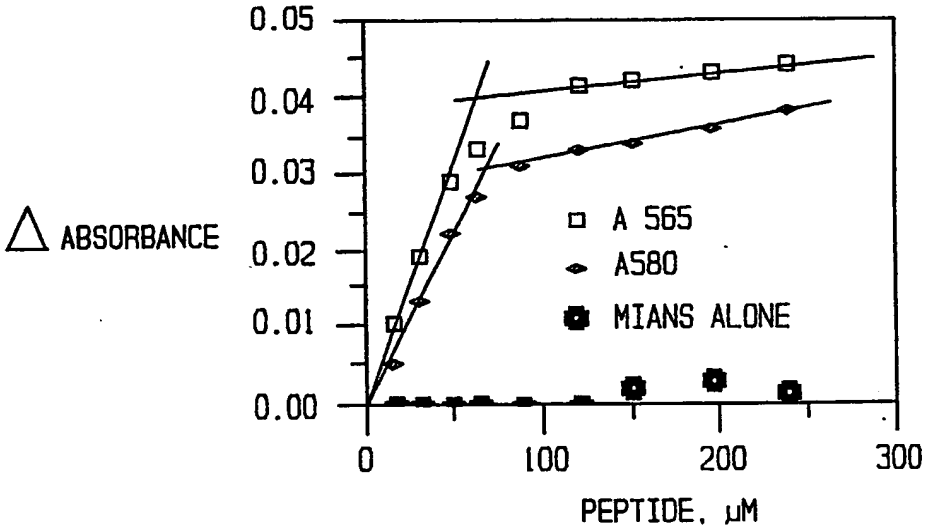


FIG.3B

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